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In re Application of:	)
	)
Yves JACOB et al.	) Group Art Unit: 1648
	)
Serial No.: 10/608,538	) Examiner: Li, Bao Q.
	)
Filed: June 30, 2003	)
	) Confirmation No.: 8265
For: CHIMERIC LYSSAVIRUS NUCLEIC ACIDS AND POLYPEPTIDES	)
	)

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Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

DECLARATION PURSUANT TO 37 C.F.R. § 1.132

I, Noël Tordo, declare that:

1. I have read and understood application Serial No. 10/608,538, including the claims, copies of which are attached hereto as Exhibit 1 and Exhibit 2, respectively;

2. I am an inventor of the subject matter in application Serial No. 10/608,538 and of the claims in Exhibit 2;

3. Pierre Perrin, Yves Jacob, Chokri Bahloul, and I, together with Corinne Jallet, Astrid Drings, and Emmanuel Desmezieres, co-authored an article entitled "Chimeric Lyssavirus Glycoproteins with Increased Immunological Potential," Journal of Virology, vol. 73, pages 225-33 (1999), a copy of which is attached hereto as Exhibit 3 and which is referred to hereinafter as "the Jallet *et al.* article";

4. I am informed and believe that this article has been cited by the U.S. patent Examiner against the claims in Exhibit 2;

5. Corinne Jallet, co-author of the Jallet *et al.* article, carried out the studies on the immune responses reported in the Jallet *et al.* article under the direction of Pierre Perrin and me;

6. Astrid Drings, a co-author of the Jallet *et al.*, article, cloned the PV-III and EBL1-PV constructs used in the experimental work reported in the Jallet *et al.* article under the direction of Pierre Perrin and me; and

7. Emmanuel Desmezieres, a co-author of the Jallet *et al.* article, participated in the cell transfections described in the Jallet *et al.* article under the direction of Pierre Perrin and me; and

8. Neither Corinne Jallet, Astrid Drings, nor Emmanuel Desmezieres made an inventive contribution to the subject matter claimed in the above-identified application.

9. The undersigned further declares that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 10 of the United States Code and that such willful false statements may jeopardize the validity of this application or any patent issuing therefrom.

Date: 26 July 2006

By: N. Tordo
Noël Tordo

Attorney Docket No. 3495.0188

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UNITED STATES PATENT APPLICATION

OF

NOËL TORDO,

PIERRE PERRIN,

YVES JACOB,

AND

CHOKRI BAHLOUL

FOR

CHIMERIC LYSSAVIRUS NUCLEIC ACIDS AND POLYPEPTIDES

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CHIMERIC LYSSAVIRUS NUCLEIC ACIDS AND POLYPEPTIDES

CROSS-REFERENCE TO RELATED APPLICATION

This application relies on, and claims the benefit of, the filing date of U.S. Provisional Application Serial No. 60/129,501, filed April 15, 1999, the disclosure of which is hereby incorporated by reference.

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to chimeric lyssavirus nucleic acids, and chimeric polypeptides and proteins encoded by these nucleic acids. More particularly, the invention relates to chimeric lyssavirus nucleic acids and proteins that can be used in immunogenic compositions, such as vaccines. Thus, the invention also relates to carrier molecules for expressing chimeric lyssavirus nucleic acids, methods of producing chimeric lyssavirus proteins and polypeptides, and methods of treating individuals to ameliorate, cure, or protect against lyssavirus infection. The compositions of the invention can also be used to express peptides, polypeptides, or proteins from organisms other than lyssaviruses. Thus, the invention provides methods of treating individuals to ameliorate, cure, or protect against many different infections, diseases, and disorders.

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Background of the Related Art

Rabies is an encephalopathic disease caused by members of the *Lyssavirus* genus within the *Rhabdoviridae* family. Rabies infects all warm-blooded animals and is almost invariably fatal in humans if not treated. On the basis of nucleotide sequence comparisons and phylogenetic analyses, the *Lyssavirus* genus has been divided into 7 genotypes (GT). GT1 includes the classical rabies viruses and vaccine strains, whereas GT2 to GT7 correspond to rabies-related viruses including Lagos bat virus (GT2); Mokola virus (GT3); Duvenhage virus (GT4); European bat lyssavirus 1 (EBL-1: GT5); European bat lyssavirus 2 (EBL-2: GT6); and Australian bat lyssavirus (GT7).

Based on antigenicity, the *Lyssavirus* genus was first divided into four serotypes. More recently, this genus was divided into two principal groups according to the cross-reactivity of virus neutralizing antibody (VNAb): Group 1 consists of GT1, GT4, GT5, GT6, and GT7, while Group 2 consists of GT2 and GT3. Viruses of group 2 are not pathogenic when injected peripherally in mice. Virulence of lyssaviruses is dependent, at least in part, on the glycoprotein present in the viral coat. Interestingly, the glycoproteins of group 2 viruses show a high degree of identity, in the region containing amino acids that play a key role in pathogenicity, to the corresponding sequence of avirulent GT1 viruses (see, for example, Coulon *et al.*, 1998, "An avirulent mutant of rabies virus is unable to infect motoneurons *in vivo* and *in vitro*", *J. Virol.* 72:273-278).

Rabies virus glycoprotein (G) is composed of a cytoplasmic domain, a transmembrane domain, and an ectodomain. The glycoprotein is a trimer, with the ectodomains exposed at the

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virus surface. The ectodomain is involved in the induction of both VNAb production and protection after vaccination, both pre- and post- exposure to the virus. Therefore, much attention has been focused on G in the development of rabies subunit vaccines. Structurally, G contains three regions, the amino-terminal (N-terminal) region, a "hinge" or "linker" region, and the carboxy-terminal (C-terminal) region. (See Figure 1.)

As depicted in Figure 1, it is generally thought that the glycoprotein (G) ectodomain has two major antigenic sites, site II and site III, which are recognized by about 72.5 % (site II) and 24 % (site III) of neutralizing monoclonal antibodies (MAb), respectively. The site II is located in the N-terminal half of the protein and the site III is located in the C-terminal half of the protein. The two halves are separated by a flexible hinge around the linear region (amino acid 253 to 257).

The G ectodomain further contains one minor site (site a), and several epitopes recognized by single MAbs (I: amino acid residue 231 is part of the epitope; V: residue 294 is part of the epitope, and VI: residue 264 is part of the epitope) (Benmansour *et al.*, 1991, "Antigenicity of rabies virus glycoprotein", *J. Virol.* 65:4198-4203; Dietzschold *et al.*, 1990, "Structural and immunological characterization of a linear virus-neutralizing epitope of the rabies virus glycoprotein and its possible use in a synthetic vaccine", *J. Virol.* 64:3804-3809; Lafay *et al.*, 1996, "Immunodominant epitopes defined by a yeast-expressed library of random fragments of the rabies virus glycoprotein map outside major antigenic sites", *J. Gen. Virol.* 77:339-346; Lafon *et al.*, 1983, "Antigenic sites on the CVS rabies virus glycoprotein: analysis with monoclonal antibodies", *J. Gen. Virol.* 64:843-845). Site II is conformational and discontinuous

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(amino acid residues 34 to 42 and amino acid residues 198 to 200, which are associated by disulfide bridges), whereas site III is conformational and continuous (residues 330 to 338). Lysine 330 and arginine 333 in site III play a key role in neurovirulence and may be involved in the recognition of neuronal receptors (see, for example, Coulon *et al.*, *supra*, and Tuffereau *et al.*, 1998, "Neuronal cell surface molecules mediate specific binding to rabies virus glycoprotein expressed by a recombinant baculovirus on the surfaces of lepidopteran cells", *J. Virol.* **72**:1085-1091). Sites II and III seem to be close to one another in the three dimensional structure and exposed at the surface of the protein (Gaudin, Y., 1997, "Folding of rabies virus glycoprotein: epitope acquisition and interaction with endoplasmic reticulum chaperones", *J. Virol.* **71**:3742-3750). However, at low pH, the G molecule takes on a fusion-inactive conformation in which site II is not accessible to MAbs, whereas sites a and III remain more or less exposed (Gaudin, Y. *et al.*, 1995, "Biological function of the low-pH, fusion-inactive conformation of rabies virus glycoprotein (G): G is transported in a fusion-inactive state-like conformation", *J. Virol.* **69**:5528-5533; Gaudin, Y., *et al.*, 1991, "Reversible conformational changes and fusion activity of rabies virus glycoprotein", *J. Virol.* **65**:4853-4859).

Moreover, several regions distributed along the ectodomain are involved in the induction of T helper (Th) cells (MacFarlan, R. *et al.*, 1984, "T cell responses to cleaved rabies virus glycoprotein and to synthetic peptides", *J. Immunol.* **133**:2748-2752; Wunner, W. et al, 1985, "Localization of immunogenic domains on the rabies virus glycoprotein", *Ann. Inst. Pasteur*, **136 E**:353-362). Based on these structural and immunological properties, it has been suggested that the G molecule may contain two immunologically active parts, each potentially able to induce

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both VNAb and Th cells (Bahloul, C. et al, 1998, "DNA-based immunization for exploring the enlargement of immunological cross-reactivity against the lyssaviruses", *Vaccine* 16:417-425).

Currently available vaccines predominantly consist of, or are derived from, GT1 viruses, against which they give protection. Many vaccine strains are not effective against GT4, and none are effective against GT2 or GT3. However, the protection elicited against GT4 through 6 depends on the vaccine strain. For example, protection from the European bat lyssaviruses (GT5 and GT6), the isolation of which has become more frequent in recent years, by rabies vaccine strain PM (Pitman-Moore) is not robust. Strain PM induces a weaker protection against EBL1 (GT5) than the protection it provides against strain PV (Pasteur virus).

Because, in part, of the importance of rabies in world health, there is a continuing need to provide safe, effective, fast-acting vaccines and immunogenic compositions to treat and prevent this disease. Many approaches other than use of whole-virus preparations have been proposed and/or pursued to provide an effective, cost-efficient immunogenic composition specific for rabies viruses. For example, as discussed above, subunit vaccines have been developed. Also, vaccines that could generate an immune response to multiple rabies serotypes as well as various other pathogens has been proposed as having some value (European Commission COST/STD-3, 1996, "Advantages of combined vaccines", *Vaccine* 14:693-700). In fact, use of a combined vaccine of diphtheria, tetanus, whole cell pertussis, inactivated poliomyelitis, and rabies has recently been reported (Lang, J. *et al.*, 1997, "Randomised Feasibility trial of pre-exposure rabies vaccination with DTP-IPV in infants", *The Lancet* 349:1663-1665). Combined vaccines including rabies have also been used for immunization of dogs (distemper, hepatitis,

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leptospirosis, and parvo-canine viruses), cats (panleukopenia, calici- and parvo-feline viruses), and cattle (foot and mouth disease virus) (Pastoret, P-P. *et al.*, 1997, "Vaccination against rabies", *In* Veterinary Vaccinology, Pastoret, P-P. *et al.*, Eds. (Elsevier): 616-628).

Moreover, vaccines produced in tissue culture are expensive to produce despite some attempts to reduce their cost. Consequently DNA vaccines, which are less expensive to produce and offer many advantages, would constitute a valuable alternative. Reports of DNA vaccinations include mouse inoculation with plasmids containing the gene encoding the rabies virus glycoprotein (G). Such inoculation is very potent in inducing humoral and cellular immune responses in association with protection against an intracerebral challenge (see, for example, Lodmell, D. *et al.*, 1998, "DNA immunization protects nonhuman primates against rabies virus", *Science Med.* 4:949-952; Xiang, Z. *et al.*, 1994, "Vaccination with a plasmid vector carrying the rabies virus glycoprotein gene induces protective immunity against rabies virus", *Virol.* **199**:132-140; and Lodmell, D. *et al.*, 1998, "Gene gun particle-mediated vaccination with plasmid DNA confers protective immunity against rabies virus infection", *Vaccine* **16**, 115). DNA immunization can also protect nonhuman primates against rabies (Lodmell et al, 1998, *supra*).

Because administration of plasmid DNA generates humoral and cellular immune responses, including cytotoxic T-Lymphocyte (CTL) production (for review see Donnelly, J. *et al.*, 1997, "DNA Vaccines", *Annu. Rev. Immunol.* **15**:617-648) and is based on a versatile technology, immunization with plasmid DNA may offer a satisfying prospect for multivalent vaccines. However, the use of a mixture of plasmids or a single plasmid expressing several antigens is believed to induce interference problems at both transcriptional and immunological

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levels (Thomson, S. *et al.*, 1998, "Delivery of multiple CD8 cytotoxic cell epitopes by DNA vaccination", *J. Immunol.* 160: 1717-1723). Therefore, there exists a need to develop and produce multivalent DNA-based vaccines that are effective against rabies and various other diseases; that are safe; and that are cost-efficient to produce and use.

BRIEF DESCRIPTION OF THE INVENTION

The present invention provides chimeric nucleic acid sequences that encode chimeric polypeptides that induce immunogenic responses in individuals (animals and humans). The nucleic acids of the invention can be expressed to provide chimeric polypeptides that elicit an immune response against rabies and/or rabies-related viruses as well as other pathogenic or otherwise undesirable organisms or polypeptides. Further, the nucleic acids of the invention themselves can elicit at least a portion of the immune response. Thus, the chimeric nucleic acids of the invention can be used to make an immunogenic composition, which can be used to treat an individual.

The present invention also provides a carrier molecule, such as a DNA expression vector, comprising the nucleic acid of the invention, which encodes a chimeric polypeptide. The carrier molecule of the invention can be used as an immunogenic composition, or as part of an immunogenic composition, to elicit the desired immune response. The desired immune response can be a protective response to rabies or rabies-related viruses as well as other organisms or polypeptides. Thus, the carrier molecules of the invention can be used to make an immunogenic

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composition, which can be used to treat an individual. The carrier molecule can also be used to produce a chimeric polypeptide.

The present invention thus provides a chimeric (fusion) protein that is encoded by the nucleic acid of the invention, or by a nucleic acid sequence present in the carrier molecule of the invention. The chimeric protein can be used to elicit an immunogenic response in an individual. The fusion protein comprises the site III antigenic determinant of a lyssavirus glycoprotein, and can comprise other antigenic sites from one or multiple other polypeptides. Thus, the chimeric polypeptide of the invention can be used to make an immunogenic composition, which can be used to treat an individual.

The present invention further provides immunogenic compositions, including vaccines, that elicit an immunological response in individuals to whom they are administered. The present invention includes immunogenic compositions comprising a polynucleotide sequence that encodes a chimeric (or fusion) polypeptide, or the chimeric polypeptide so encoded, which elicits the immune response. The immunogenic compositions and vaccines of the present invention provide an increased level of immune stimulation and enhanced protection against rabies viruses, and broaden the spectrum of protection against rabies-related viruses, as compared to immunogenic compositions known in the art. The immunogenic compositions also provide multiple immunogenic active sites for induction of an immune response against rabies epitopes as well as epitopes unrelated to rabies.

In view of the above embodiments of the invention, it is evident that the present invention also provides a method of producing a chimeric nucleic acid, a method of producing a carrier

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molecule, a method of producing a fusion protein, and a method of making an immunogenic composition, such as a vaccine. The immunogenic composition so made can be used to treat (*e.g.*, immunize) individuals.

Included in the invention is the use of the nucleic acid, polypeptide, and/or carrier molecule of the invention to elicit an immune response, such as a protective immune response. Thus, the invention includes the use of a vaccine comprising the polynucleotide, polypeptide, and/or carrier molecule of the invention to treat an individual, either prophylactically or therapeutically. Therefore, the invention includes prophylactic treatment methods, therapeutic treatment methods, and curative treatment methods.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will be described in greater detail with reference to the drawings, in which:

Figure 1 depicts lyssavirus glycoproteins and chimeric constructs of lyssavirus glycoproteins. SP - Signal peptide; TM - Transmembrane domain; S_1 - NL amino acid residues; S_2 - NS amino acid residues; S_3 - LFAV amino acid residues.

(A) A schematic representation of lyssavirus PV glycoprotein (G) is presented at the top, indicating regions encompassing site II, site III, and epitopes I, V, and VI, as well as the transmembrane domain (TM). Chimeric polypeptides are schematically depicted on the other lines, with deletion and/or fusion points indicated by residue numbers. Numbers are the positions of amino acid residues on the mature protein (signal peptide cleaved).

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(B) Amino acid sequences of the chimeric polypeptides at the fusion sites. The linear region carrying epitope VI (amino acid 264) is indicated by underlining at residues 251-275. Black and gray boxes outline the EBL-1 and Mok sequences, respectively. Dashes represent amino acids similar to those of the PV sequence, and dots represent gaps.

(C) Schematic representation of the inserted sequences encoding the C3 poliovirus epitope involved in virus neutralizing antibody induction (B), and lymphocytic choriomeningitis virus (LCMV) nucleoprotein CD8 H-2^d (CTL) cell epitope involved in the induction of both cytotoxic T Lymphocytes (CTL) and protection against LCMV challenge in the truncated (GPVIII) and chimeric lyssavirus G protein (GEBL1-PV).

(D) Putative PEST sequence analysis around the junction of the end of EBL1 part and the beginning of B cell epitope is also reported.

(E) Comparison of the deduced amino acid sequences of G proteins of selected lyssaviruses. The consensus sequence is presented as the bottom sequence. Light grey boxes indicate the main antigenic sites. Dark grey boxes indicate the hydrophobic signal peptide (SP) and the transmembrane domain (TM). Underlined NX(S/T) motifs are potential N-glycosylation sites.

Figure 2 shows indirect immunofluorescence microscopy of lyssavirus G production in Neuro-2a cells 48h after transient transfection with various plasmids: pGPV-PV (A, B and C), pG-PVIII (D, E and F), pGEBL1-PV (G, H, and I), pGMok-PV (J, K, and L), and pGPV-Mok (M, N, and O). Forty-eight hours after transfection, cells were permeabilized and stained with antibodies: anti-PV G PAb (A, D, G, J, and M), PV D1 anti-native PV G site III MAb (B, E, H,

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and K), 6B1 anti-denatured G site III MAb (C and F), anti-EBL-1 G PAb (I), anti-Mok G PAb (L and N) and serum from an unimmunized mouse (O).

Figure 3 shows the results of a kinetic study of antigen synthesis in Neuro-2a cells transiently transfected with pGPV-PV (A), pGEBL1-PV (B), or pG-PVIII (C). Cells were permeabilized at various times and stained with PV PAb (white bar) or anti-denatured G site III 6B1 MAb (black bar).

Figure 4 shows the results of induction of IL-2-producing cells by plasmids encoding various lyssavirus G. BALB/c mice (two animals for each plasmids) were injected i.m. (50 μl in each anterior tibialis muscle) with 40 μg plasmid (pGPV-PV, pG-PVIII, pGEBL1-PV, pGMok-PV and pGPV-Mok). Spleens were removed 21 days later and splenocytes were specifically stimulated *in vitro* by inactivated and purified viruses (PV, EBL1 or Mok), G PV, or polyclonally stimulated by concanavalin A (ConA). The amount of IL-2 released was then assayed in triplicates by bioassay and titers expressed as U/ml.

Figure 5 shows induction of VNAb against European lyssavirus genotypes by plasmid. BALB/c mice were injected with 40 μg plasmid in the tibialis muscle.

(A) Injection with pGPV-PV. Mice received a boost on day 30. Sera (pool of 3 samples) were assayed on days 27 and 40 for VNAb against viruses of genotypes 1 (CVS and PV), 5 (EBL1b), and 6 (EBL2b).

(B) Injection with pGEBL1-PV. Four mice received only one injection of plasmid and blood samples were collected at various intervals by trans-orbital puncture. Sera were assayed by RFFIT using PV and EBL1b viruses for VNAb determination.

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Figure 6 shows comparative protection induced by pGPV-PV, pGEBL1-PV plasmids, and rabies PM and PV vaccines against CVS, EBL-1b, and EBL-2b. BALB/c mice (9 animals per series) were injected i.p. on days 0 and 7 with 0.5 ml of PM vaccine diluted 1/10th (solid circles) or with 2 µg of inactivated and purified PV virus (solid squares). For DNA-based immunizations, BALB/c mice (5 animals for each plasmid) were injected in the tibialis muscle with PBS (open circles) or with 40 µg of various plasmids pGPV-PV (diamond), EBL1-PV (solid triangle), pCIneo backbone (cross). Swiss mice (6 animals) were injected with pGPV-PV (open square).

(a) Inactivated virus was injected. BALB/c and Swiss mice were challenged i.c. on day 21 with about 30 LD_{50} of CVS.

(b) Inactivated virus was injected. BALB/c and Swiss mice were challenged i.c. on day 21 with about 30 LD_{50} of EBL-1b.

(c) Inactivated virus was injected. BALB/c and Swiss mice were challenged i.c. on day 21 with about 30 LD_{50} of EBL-2b.

(d) DNA was injected. BALB/c and Swiss mice were challenged i.c. on day 21 with about 30 LD_{50} of CVS.

(e) DNA was injected. BALB/c and Swiss mice were challenged i.c. on day 21 with about 30 LD_{50} of EBL-1b.

(f) DNA was injected. BALB/c and Swiss mice were challenged i.c. on day 21 with about 30 LD_{50} of EBL-2b.

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Figure 7 shows indirect immunofluorescence microscopy of antigens expressed in Neuro-2a cells transfected with plasmids.

(A) Cells were transfected with plasmid pGEBL1-(B-CTL)$_2$-PV and stained with the rabies D1 MAb.

(B) Cells were transfected with plasmid pGEBL1-(B-CTL)$_2$-PV and stained with the poliovirus C3 MAb.

(C) Cells were transfected with plasmid pGEBL1-(B-CTL)$_2$-PV and stained with the anti-poliovirus type 1 PAb.

(D) Cells were transfected with plasmid pCIneo and stained with either 1) the rabies D1 MAb, 2) the poliovirus C3 MAb, or 3) the anti-poliovirus type 1 PAb.

Figure 8 shows induction of IL-2-producing cells by pGPVIII (A) or pGEBL1-PV (B) carrying poliovirus and LCMV epitopes. BALB/c mice (two animals for each plasmids) were injected i.m. (50 µg in each anterior tibialis muscle) with plasmids. Spleens were removed 14 days later and splenocytes were stimulated *in vitro* by cell culture medium (solid bar) specifically by inactivated and purified lyssaviruses (IPLV PV: hashed bar; IPLV EBL: brick) or polyclonally stimulated by ConA (open bar). The amount of IL-2 released was then assayed in triplicates by bioassay and titers expressed as U/ml.

(A) Plasmids injected were pCIneo -empty plasmid-, pGPVIII, and p(B-CTL)$_2$-GPVIII.

(B) Plasmids injected were pCIneo, pGEBL1-PV, pGEBL1-(B)-PV, pGEBL1-(CTL)-PV, pGEBL1-(CTL-B)-PV, pGEBL1-(B-CTL)$_2$-PV.

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Figure 9 shows a kinetic study in BALB/c mice of antibody production induced by p(B-CTL)2-GPVIII or pGPVIII against poliovirus peptide and rabies virus. Three mice were injected with 40 µg of p(B-CTL)2-GPVIII (square and circle), or pGPVIII (triangle), or empty pCIneo (diamond). After puncture by retro-orbital route at various times, sera were assayed by ELISA for the determination of antibody against poliovirus peptide (square and diamond) or rabies virus (circle, triangle and diamond).

Figure 10 shows the influence of a priming on the poliovirus anti-peptide antibody production induced by p(B-CTL)2-GPVIII. Five groups of three mice received on day 0 PBS (2 groups), p(B-CTL)2-GPVIII (2 groups), or pPVIII. One group (injected with p(B-CTL)2-GPVIII) was not boosted, whereas the group injected with pPVIII was boosted with p(B-CTL)2-GPVIII on day 26. One group (injected with p(B-CTL)2-GPVIII) was boosted with p(B-CTL)2-GPVIII on day 14. All animals were controlled for anti-peptide antibody production on day 39 by ELISA.

Figure 11 shows the production of rabies virus neutralizing antibodies against the challenge virus standard (EVS) after injection of the full homogeneous plasmid pGPV in beagle dogs.

Group A: Injection of 100 µg of plasmid in one site on days, 0, 21, 42, and 175.

Group B: Injection of 33 µg of plasmid in three sites on days 0, 21, 42, and 175.

Group C: Injection of 100 µg of plasmid in one site on days 0 and 175.

Group D: Injection of phosphate buffered saline (control).

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Figure 12 shows individual neutralizing antibody response against a wild rabies virus (fox from France, fox wild rabies virus FWR) after injection of the full homogeneous plasmid pGPV in beagle dogs. It shows also the protection induced against an intramuscular challenge performed on day 175 with a wild rabies virus isolated from rabid dogs.

Dogs nos. 1 to 3	=	group A
Dogs nos. 4 to 6	=	group B
Dogs nos. 7 to 9	=	group C
Dogs nos. 10 to 12	=	group D

Figure 13 shows the results of indirect immunofluorescence of N_2A cells transfected with plasmid DBL-3/PVIII (Fugene, Roche Molecular Biochemicals) and fixed with acetone (80%) for 10 minutes on ice.

(A) Results of mouse anti-DBL-3 GTST fusion protein (1/1000 dilution) are shown in the top panel. Background reactivity seen with the second antibody (anti-mouse) conjugated to FITC is shown in the bottom panel.

(B) Results of rabbit anti-PVIII (1/400 dilution) are shown in the top panel. Background reactivity seen with the second antibody (anti-rabbit) conjugated to FITC is shown in the bottom panel.

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

The inventors have created chimeric nucleic acid sequences that encode chimeric polypeptides that induce immunogenic responses in individuals. As discussed above, it is known

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in the art that nucleic acids can elicit both humoral and cellular immune responses. In doing so, it is possible for the nucleic acids not only to indirectly induce an immune response via an encoded peptide, polypeptide, or protein, but for the nucleic acids to directly induce an immune response. Thus, the nucleic acids of the invention can themselves induce at least part of the immunogenic response. Accordingly, according to the invention, the chimeric nucleic acids and carrier molecules can directly or indirectly provide or promote an immunogenic response when used according to the invention.

As used herein, "chimeric" and "fusion" are used interchangeably and in reference to both nucleic acids and polypeptides. These terms refer to nucleic acids and polypeptides that comprise sequences that are not found naturally associated with each other in the order or context in which they are placed according to the invention. For example, a chimeric glycoprotein can comprise a C-terminal region from a rabies GT1 and an N-terminal region from a rabies GT3 or GT5. Further, a chimeric nucleic acid can comprise a short segment from one portion of a rabies genotype linked directly to another segment from the same genotype, where the two segments are not naturally adjacent each other. Thus, a chimeric or fusion nucleic acid or polypeptide does not comprise the natural sequence of a rabies virus in its entirety, and may comprise heterologous (from another strain of lyssavirus, or from another organism altogether) sequences. Fusion/chimeric proteins have the two (or more) segments joined together through normal peptide bonds, while fusion/chimeric nucleic acids have the two (or more) segments joined together through normal phosphodiester bonds.

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In one aspect of the invention, the chimeric nucleic acids comprise a) a sequence encoding site III of a glycoprotein, b) a sequence encoding the transmembrane domain (or a portion thereof that is functionally equivalent to the transmembrane domain) of a glycoprotein, and c) a sequence that encodes the cytoplasmic domain of the glycoprotein of a lyssavirus. In preferred embodiments of this aspect of the invention, the chimeric nucleic acids further comprise a sequence encoding site II of a lyssavirus glycoprotein. In embodiments, the sequence encoding the transmembrane domain (or portion thereof) is a sequence from a transmembrane glycoprotein of a lyssavirus. In other embodiments, the sequence is from a transmembrane glycoprotein other than a glycoprotein from a lyssavirus. For example, it can be from a glycoprotein from another organism, or from a transmembrane protein that is not a glycoprotein. In embodiments, the sequence encoding the transmembrane domain (or portion thereof) and the sequence encoding the cytoplasmic domain are from the same lyssavirus. In embodiments of this aspect of the invention, the sequence encoding site III and the cytoplasmic domain are from the same protein, preferably a lyssavirus protein, such as a lyssavirus glycoprotein.

In a preferred embodiment of this aspect of the invention, the chimeric nucleic acid includes sequences that encode a site III sequence of a lyssavirus glycoprotein, a site II sequence of a lyssavirus glycoprotein, a transmembrane domain of a transmembrane protein, and a cytoplasmic domain of a lyssavirus glycoprotein. The transmembrane domain can be from a lyssavirus or another organism. It is preferred that the chimeric nucleic acid of the invention comprise sequences encoding an antigenic protein (or antigenic portion thereof), especially between the sequences encoding sites II and III.

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In preferred embodiments, this aspect of the invention provides a polynucleotide comprising a polynucleotide sequence, wherein the polynucleotide sequence comprises a sequence encoding site III of a lyssavirus glycoprotein, and wherein the polynucleotide sequence does not comprise a sequence encoding an entire lyssavirus glycoprotein.

The sequences encoding site II and site III are sequences encompassing site II and site III of a glycoprotein of a lyssavirus, respectively. In embodiments, the sequence encoding site III is not identical to any of the known site III sequences of lyssaviruses, but shows at least 60% identity with one of the lyssavirus sequences, extending 30 bp on each side of the site III sequence. Sequence analysis and phylogenetic studies are performed using various packages: GCG (version 8.1, 1994), CLUSTAL W (Thompson, 1994 #1040), PHYLIP (Version 3.5: Felseustein, 1993, #1042), and GDE (Genetic Data Environment, Version 2.2: Institut Pasteur Scientific Computer Service - S.I.S.).

In preferred embodiments of this aspect of the invention, the site III sequence is from rabies virus strain PV, or shows at least 60% identity to that site III sequence. Highly satisfactory results are obtained with the following constructs, displayed in Figure 1: PV-PV or PV III or EBL1-PV or MOK-PV. In highly preferred embodiments, the site III sequence is a PV III sequence.

The inventors have found that the presence of site III of the lyssavirus glycoproteins improves the immunogenicity of compositions comprising the glycoprotein, or portions thereof. Thus, the present invention includes chimeric nucleic acids that comprise a sequence encoding site III of a lyssavirus glycoprotein that is functionally, operatively, and physically linked to a

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homologous or heterologous sequence encoding a transmembrane domain from natural or synthetic sequence encoding a transmembrane protein (or a portion thereof that is functionally equivalent to the transmembrane domain), and a sequence encoding a cytoplasmic domain (or a portion thereof that is sufficient to stably exist cytoplasmically) from a glycoprotein. Preferably, the glycoprotein is that of a virus and particularly that of a lyssavirus. The chimeric nucleic acid sequences can all be from the same lyssavirus, can be selected from various lyssaviruses, or can be from both lyssaviruses and other viruses and organisms. In preferred embodiments, the nucleic acid sequences encoding the site III and the cytoplasmic domain are from the same lyssavirus.

In addition, the chimeric nucleic acid of the invention can comprise a sequence that encodes an antigenic polypeptide, or an antigenic portion thereof, from another virus or organism (a heterologous sequence). For example, the chimeric nucleic acid can comprise, in addition to the elements set forth above, a sequence that encodes an epitope from leishmania, diphtheria, tetanus, poliomyelitis, foot and mouth disease virus, herpes viruses, canine distemper viruses, parvovirus, and feline immunodeficiency virus. Alternatively, or in addition, the chimeric nucleic acid can comprise a sequence that encodes a tumor antigen. The sequence encoding the heterologous polypeptide (or antigenic portion thereof) is fused (in frame) with the coding sequence detailed above, at any site that results in a functional product. In this way, the chimeric nucleic acid of the invention provides the coding sequence for multiple antigenic determinants, including, but not limited to, rabies virus epitopes.

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The chimeric nucleic acids of the invention can be used to make an immunogenic composition. The immunogenic composition can consist essentially of the chimeric nucleic acid or can comprise the chimeric nucleic acid in addition to other components, including, but not limited to, adjuvants, excipients, stabilizers, supra molecular vectors such as those described in European Patent No. 696,191 (Samain *et al.*), and antigens. The components typically included in immunogenic compositions are well known to the skilled artisan in the field, as are the techniques for preparation of immunogenic compositions, such as vaccines. Therefore, preparation of the immunogenic composition can be achieved by the skilled artisan using well known techniques without undue or excessive experimentation. In embodiments, the immunogenic compositions of this aspect of the invention induce humoral immunity, cellular immunity, or both.

In another aspect of the invention, the chimeric nucleic acid of the invention is present as part of a carrier molecule. The core of the carrier molecule can be any molecule that is known to be useful to maintain, and preferably express, a heterologous peptide or polypeptide-encoding nucleic acid. The core of the carrier molecule can be, for example, a plasmid, a phage, a phagemid, a cosmid, a virus, a yeast artificial chromosome (YAC), or the like. Such core carrier molecules are also commonly referred to as vectors or expression vectors, and are well-known to the skilled artisan and are widely available to the public. The carrier molecule of the invention can be provided as a naked nucleic acid, or packaged, such as in a viral shell or coat. The carrier molecule can be provided as DNA or RNA. Modified forms of these two nucleic acids are included within the scope of the invention. Preferably, the carrier molecule comprises sequences

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that permit transcription of the chimeric nucleic acids of the invention. These sequences are operably linked to the chimeric nucleic acids of the invention (*i.e.* their operation/function directly affects expression of the chimeric nucleic acid). In embodiments, these sequences include regulatory elements that allow controlled expression of the chimeric nucleic acids so that expression of the chimeric nucleic acids can be regulated, by, for example, delaying expression until desired or expressing the chimeric nucleic acids in certain tissues or cell types only. Such control elements are known to the artisan in the field and can routinely be inserted or removed from the carrier molecules as desired or necessary using well-known molecular biology techniques and reagents.

In embodiments, the carrier molecule contains a polynucleotide sequence that comprises a sequence encoding a part of a glycoprotein containing at least the site III of a lyssavirus glycoprotein. In embodiments, the carrier molecule contains a polynucleotide sequence that comprises a) a sequence encoding a part of a glycoprotein containing at least the site III of a lyssavirus glycoprotein, and b) a sequence encoding a transmembrane domain of a transmembrane protein. In embodiments, the carrier molecule contains a polynucleotide sequence that comprises a sequence encoding at least the C-terminal half of a lyssavirus glycoprotein. In embodiments, the carrier molecule contains a polynucleotide sequence that comprises a) a sequence encoding at least the C-terminal half of a lyssavirus glycoprotein, and b) a sequence encoding a transmembrane domain of a transmembrane protein. In embodiments, the carrier molecule contains a polynucleotide sequence that comprises a) a sequence encoding at least the C-terminal half of a lyssavirus glycoprotein, b) a sequence encoding a transmembrane

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domain of a transmembrane protein, and c) a sequence encoding cytoplasmic domain of the lyssavirus glycoprotein.

In an embodiment of the invention, a carrier molecule according to the invention comprises nucleic acids encoding a) the site III of the glycoprotein of a lyssavirus, b) a transmembrane domain of a glycoprotein (or a portion thereof that is functionally equivalent to the transmembrane domain), and c) a sequence that encodes the cytoplasmic domain of the glycoprotein of a lyssavirus. In preferred embodiments of this aspect of the invention, the carrier molecule further comprises a sequence encoding site II of a lyssavirus glycoprotein. In embodiments, the sequence encoding the transmembrane domain (or portion thereof) is a sequence from the glycoprotein of a lyssavirus. In other embodiments, the sequence is from a transmembrane glycoprotein other than a glycoprotein from a lyssavirus. In embodiments, the sequence encoding the transmembrane domain (or portion thereof) and the sequence encoding the cytoplasmic domain are from the same lyssavirus. In embodiments, the sequence encoding the site III and the cytoplasmic domain are from the same lyssavirus.

In a preferred embodiment, the carrier molecule further comprises at least one antigenic sequence other than site III or site II of a lyssavirus. The additional antigenic sequence(s) can be from any organism and includes, but is not limited to, antigenic sequences from parasites (for example leishmania), bacteria, viruses, and tumor cells. The carrier molecule of the present invention, by providing the region of lyssavirus glycoprotein required for enhanced immunogenicity (site III), allows the production of a high level immune response to not only the lyssavirus antigens (sites III and II), but to the heterologous antigen(s) fused to the lyssavirus

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sequences. The carrier molecule can thus be used to elicit an immune response to multiple antigens from different organisms.

The carrier molecule of the invention preferably comprises a chimeric sequence that encodes a chimeric polypeptide that comprises at least one antigenic determinant, which is site III of a lyssavirus. More preferably, the carrier molecule comprises a chimeric sequence that encodes a chimeric polypeptide that comprises the site III of a lyssavirus and at least one other antigenic determinant selected from the group consisting of an antigen from the same lyssavirus from which the site III was derived and a heterologous antigen. The other antigenic determinants include, but are not limited to, those of pathogenic parasites (*e.g., Plasmodium*), viruses, bacteria, and those of tumor cells.

A preferred carrier molecule according to the invention is pEBL1-PV, which was deposited at the Collection Nationale de Cultures de Microorganismes (CNCM), located at Institut Pasteur, 28, Rue du Docteur Roux, F-75724 Paris, Cedex 15, France, on December 22, 1998, in accordance with the Budapest Treaty, and which was accorded Accession Number I-2114. Another preferred carrier molecule according to the invention is pVIII, which was deposited at the CNCM on December 22, 1998, in accordance with the Budapest Treaty, and which was accorded Accession Number I-2115.

The carrier molecules of the invention can be used to make an immunogenic composition. The immunogenic composition can consist essentially of the carrier molecule or can comprise the carrier molecule in addition to other components, including, but not limited to, adjuvants, excipients, stabilizers, supra molecular vectors (EP 696,191, Samain *et al.*), and antigens. The

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components typically included in immunogenic compositions are well known to those skilled in the field, as are the techniques for preparation of immunogenic compositions, including vaccines. Preparation of the immunogenic composition is a routine matter that can be achieved by the skilled artisan using well-known techniques without undue or excessive experimentation and thus need not be described in detail herein. In embodiments, the immunogenic compositions of this aspect of the invention induce humoral immunity, cellular immunity, or both.

In another aspect, the present invention provides a chimeric polypeptide or protein that is encoded and/or expressed by the chimeric nucleic acid and/or carrier molecule of the present invention. In embodiments, the chimeric polypeptide comprises a) site III of a lyssavirus glycoprotein, b) a transmembrane domain of a glycoprotein (or a functional portion thereof), and c) a cytoplasmic domain of the glycoprotein of a lyssavirus. In preferred embodiments of this aspect of the invention, the chimeric polypeptides further comprise site II of a lyssavirus glycoprotein. In embodiments, the transmembrane domain (or portion thereof) is from the glycoprotein of a lyssavirus. In other embodiments, the transmembrane domain (or portion thereof) is from a transmembrane glycoprotein other than a glycoprotein from a lyssavirus. In other embodiments, the transmembrane domain (or portion thereof) and the cytoplasmic domain are from the same lyssavirus. In embodiments, the site III and the cytoplasmic domain are from the same lyssavirus.

Site II and site III can be obtained from any lyssavirus, and both can, but do not necessarily, come from the same lyssavirus. Further, the sequence of site II and/or site III does not have to be identical to a site II or site III of a glycoprotein of a lyssavirus. In embodiments,

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the sequence of one or both is not identical to any of the known site II or site III sequences of lyssaviruses, but shows at least 60% identity with one of the lyssavirus sequences, extending 10 residues on each side of the site II or site III sequence.

In preferred embodiments, the chimeric polypeptide of the invention further comprises at least one antigenic determinant other than lyssavirus glycoprotein site III or site II. The other antigenic determinant can be an antigenic protein, or antigenic portion thereof, from another rabies virus or rabies related virus, from another virus, from a parasite, a bacterium, or any other organism or cell that expresses an undesirable antigenic determinant. In embodiments, the other antigenic determinant is from a tumor cell. In embodiments, the other antigenic determinant is from a parasite that causes malaria, such as *Plasmodium falciparum* or *Plasmodium vivax*.

Thus, the invention provides a chimeric polypeptide comprising, as the only antigenic determinant, site III of a lyssavirus. Because antigenicity is dependent, at least to some extent, on the individual to whom the immunogenic composition is administered, a chimeric polypeptide having only one antigenic determinant in one organism may have more than one antigenic determinant in another. However, according to the invention, if a chimeric polypeptide has only one antigenic determinant in at least one individual, regardless of the number it has in other individuals, it is a chimeric polypeptide according to this embodiment of the invention.

The invention also provides a chimeric polypeptide with multiple antigens including, but not limited to, rabies antigens. The chimeric polypeptide can be used as, or as part of, an immunogenic composition, such as a vaccine. Thus, the polypeptide of the invention is an immunogenic polypeptide that contains at least one region (which can be isolated as a fragment)

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that induces an immunogenic response. In embodiments where a site II, a site III, and another antigenic determinant are present, it is preferably, but not necessary, for the other antigenic determinant to be located between site II and site III in the linear (primary) amino acid sequence of the polypeptide. A preferred antigen other than site II or site III is a tumor antigen from a tumor cell. In embodiments, the polypeptides or proteins of the invention induce humoral immunity, cellular immunity, or both.

Preferably, the chimeric nucleic acids, carrier molecules, and chimeric polypeptides (the "molecules") of the invention are isolated and/or purified. The terms "isolated" and "purified" refer to a level of purity that is achievable using current technology. The molecules of the invention do not need to be absolutely pure (*i.e.*, contain absolutely no molecules of other cellular macromolecules), but should be sufficiently pure so that one of ordinary skill in the art would recognize that they are no longer present in the environment in which they were originally found (*i.e.*, the cellular milieu). Thus, a purified or isolated molecule according to the invention is one that has been removed from at least one other macromolecule present in the natural environment in which it was found. More preferably, the molecules of the invention are essentially purified and/or isolated, which means that the composition in which they are present is almost completely, or even absolutely, free of other macromolecules found in the environment in which the molecules of the invention are originally found. Isolation and purification thus does not occur by addition or removal of salts, solvents, or elements of the periodic table, but must include the removal of at least one macromolecule.

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As can be seen from the above disclosure, the invention provides an immunogenic composition. The immunogenic composition can comprise the chimeric nucleic acid, chimeric protein, and/or carrier molecule of the invention. For example, the chimeric polypeptides of the invention can be used to make an immunogenic composition. The immunogenic composition can consist essentially of the chimeric polypeptide or can comprise the chimeric polypeptide in addition to other components, including, but not limited to, adjuvants, excipients, stabilizers, supra molecular vectors (EP 696,191, Samain *et al.*) and antigens. The components typically included in immunogenic compositions are well known to those skilled in the field, as are the techniques for preparation of immunogenic compositions, such as vaccines. Therefore, preparation of the immunogenic composition can be achieved by the skilled artisan using well known techniques without undue or excessive experimentation.

The immunogenic composition according to the invention is a composition that elicits an immune response at least to a lyssavirus. Because the chimeric nucleic acids (and thus carrier molecules and polypeptides) of the invention can comprise antigenic determinants from the various lyssavirus genotypes in various combinations, the immunogenic composition of the invention can provide, or elicit, a broad spectrum of protection against lyssaviruses that induce encephalomyelitis, including rabies viruses. Furthermore, because sequences encoding multiple lyssavirus epitopes can be included in one chimeric nucleic acid, the immunogenic composition of the invention can provide an immune response to multiple (including all) genotypes of lyssavirus. Preferably, the immunogenic composition of the invention elicits both a cellular and a humoral immune response.

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In addition, the immunogenic composition of the invention can provide epitopes from not only lyssaviruses, but from any other organism as well (including antigens produced by human cells, such as undesirable antigens found on the surface of cancerous cells). This permits the construction of immunogenic compositions, including, but not limited to vaccines, having broad applicability in that a single composition can be used to elicit an immune response to multiple pathogens. For example, an immunogenic composition can be made that provides a protective immunological response to a broad range of lyssaviruses while at the same time providing a protective response to other viruses such as polio and influenza. Such a multivalent immunogenic composition is provided by the chimeric nature of the nucleic acids and polypeptides of the invention, as well as the presence of site III of a lyssavirus, which confers a strong immunogenic response to the epitopes of the antigenic polypeptide.

The immunogenic composition of the invention elicits an immunogenic response in individuals to whom it is administered. The immunogenic response can elicit a protective immune response, but such a response is not necessary. According to the invention, immunogenic compositions that elicit a protective response are referred to as vaccines. The immunogenic responses can be enhanced or otherwise modified by the inclusion of components, in addition to the chimeric nucleic acids, chimeric proteins, and carrier molecules of the invention. Alternatively, the immunogenic compositions can consist essentially of the chimeric nucleic acids, chimeric proteins, and carrier molecules of the invention. Thus, the invention encompasses DNA vaccines comprising the chimeric nucleic acids and/or the carrier molecules of the invention.

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The invention thus provides a method of making an immunogenic composition. In one embodiment, the method comprises isolating and/or purifying the chimeric nucleic acid or polypeptide or the carrier molecule. In another embodiment, the method comprises isolating and/or purifying the nucleic acid or polypeptide or the carrier molecule, then combining it with additional components. The additional components can be any suitable compound that does not have an adverse effect on the immunogenicity, safety, or effectiveness of the nucleic acid, polypeptide, or carrier molecule of the invention. The additional components include, but are not limited to, compounds and additives that are typically added to immunogenic compositions to enhance immunogenicity, stability, and bioavailability. Exemplary additives are disclosed above and are well known to those skilled in the art.

In another embodiment of this aspect of the invention, the method of making an immunogenic composition is a method of expressing a chimeric (hybrid) polypeptide for use in the production of an immunogenic composition. In this embodiment, the chimeric nucleic acid or carrier molecule of the invention is expressed (transcribed and translated) so that the chimeric polypeptide is produced. The chimeric polypeptide so produced is then isolated and/or purified to an acceptable level so that it can be used to make an immunogenic composition. Production of an immunogenic composition in this embodiment of the invention is according to the disclosure herein. As used herein, a polypeptide is a polymer of amino acids and includes peptides (more than 3 amino acids in length), and proteins (more than 100 amino acids in length). Production of the chimeric polypeptide can be performed *in vivo* or *in vitro*. Preferably, production occurs *in vivo* by expression in bacterial or tissue cultures, and the chimeric

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polypeptide is isolated or purified from those cultures using known protein purification techniques.

In a further aspect of the invention, methods of making the chimeric nucleic acid, carrier molecules, and chimeric polypeptides of the invention are provided. The methods include commonly known genetic engineering techniques that are well-known to the skilled artisan. Any known technique that is routinely practiced by the skilled artisan can be used to produce and purify the chimeric molecules of the invention. The novelty of the invention does not lie in these techniques, but in the chimeric molecules constructed through the use of them. In this aspect, the methods can be used to make a nucleic acid or carrier molecule for use in the production of an immunogenic composition, such as a DNA vaccine. The invention thus includes a method of making a composition for use in a DNA vaccine.

The invention also provides methods of treating individuals with the immunogenic compositions of the invention. The methods of treating can be methods of therapy or prophylaxis. In embodiments, a method of treating is provided wherein the method induces an immune response in an individual (an animal or a human). In embodiments, the method of treating is a method of inducing a protective response, *i.e.*, a method of vaccination. In other embodiments, the method of treating is a method of inducing an immune response in an individual already suffering from a disease or disorder, such as an infection or cancer. Preferably, the method is a method of vaccination.

The method of treating comprises administering the immunogenic compositions to individuals, or patients, in need of treatment, suspected of needing treatment, or desiring

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prophylactic (protective) treatment for a disease or disorder. Any known method of administration can be used in this aspect of the invention, including, but not limited to, injection with syringe and needle (*e.g.*, subcutanaceous, intramuscular, intravenous), oral or mucosal administration, inhalation, topical administration(*e.g.*, applying directly to the skin), and by suppository.

In an embodiment of this aspect of the invention, the method comprises administering the chimeric nucleic acids of the invention to an individual in an amount sufficient to elicit an immunogenic reaction (immune response) in the recipient. Preferably, this response is a protective response. The amount of nucleic acid necessary for such an immunization can be determined by those of skill in the art without undue or excessive experimentation. For example, compositions comprising the chimeric nucleic acids and carrier molecules of the invention can be administered in an amount of 40 to 100 μg intramuscularly in one or several injections. A 100 μg dosage is generally useful for dogs, and a 40 μg dosage for mice (weight 20 g).

In another embodiment of this aspect of the invention, the method comprises administering the chimeric polypeptides of the invention to an individual in an amount sufficient to elicit an immunogenic reaction (immune response) in the recipient. Preferably, the response is a protective response. The amount of polypeptide necessary for such an immunization can be determined by those of skill in the art without undue or excessive experimentation. For example, compositions comprising the chimeric polypeptides of the invention can be administered in an amount of 1 to 10 μg intramuscularly in one or several injections.

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In another embodiment of this aspect of the invention, the method comprises administering the carrier molecule of the invention to an individual in an amount sufficient to elicit an immunogenic reaction (immune response) in the recipient. Preferably, the response is a protective response. The amount of carrier molecule necessary for such an immunization can be determined by those of skill in the art without undue or excessive experimentation. For example, compositions comprising the carrier molecules of the invention can be administered in an amount of 40 to 100 μg intramuscularly, in one or several injections.

Thus, this aspect of the invention provides a method of DNA vaccination. The method also includes administering any combination of the chimeric nucleic acids, the chimeric polypeptides, and the carrier molecule of the invention to an individual. In embodiments, the individual is an animal, and is preferably a mammal. Preferably, the mammal is selected from the group consisting of a human, a dog, a cat, a bovine, a pig, and a horse. In a preferred embodiment, the mammal is a human. In a preferred embodiment, the mammal is a dog. In a preferred embodiment, the mammal is a horse.

The methods of treating include administering immunogenic compositions comprising not only polypeptides, but compositions comprising nucleic acids (including the carrier molecule) as well. Those of skill in the art are cognizant of the concept, application, and effectiveness of nucleic acid vaccines (*e.g.*, DNA vaccines) and nucleic acid vaccine technology as well as protein and polypeptide based technologies. The nucleic acid based technology allows the administration of nucleic acids, naked or encapsulated, directly to tissues and cells without the need for production of encoded proteins prior to administration. The technology is based on

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the ability of these nucleic acids to be taken up by cells of the recipient organism and expressed to produce an immunogenic determinant to which the recipient's immune system responds. Typically, the expressed antigens are displayed on the cell surface of cells that have taken up and expressed the nucleic acids, but expression and export of the encoded antigens into the circulatory system of the recipient individual is also within the scope of the present invention. Such nucleic acid vaccine technology includes, but is not limited to, delivery of naked DNA and RNA, and delivery of expression vectors encoding polypeptides of interest (carrier molecules). Although the technology is termed "vaccine", it is equally applicable to immunogenic compositions that do not result in a protective response. Such non-protection inducing compositions and methods are encompassed within the present invention.

Although it is within the present invention to deliver nucleic acids and carrier molecules as naked nucleic acid, the present invention also encompasses delivery of nucleic acids as part of larger or more complex compositions. Included among these delivery systems are viruses, virus-like particles, or bacteria containing the chimeric nucleic acid or carrier molecule of the invention. Also, complexes of the invention's nucleic acids and carrier molecules with cell permeabilizing compounds, such as liposomes, are included within the scope of the invention. Other compounds, supra molecular vectors (EP 696,191, Samain *et al.*) and delivery systems for nucleic acid vaccines are known to the skilled artisan, and exemplified in WO 90 111 092 and WO 93 06223, and can be made and used without undue or excessive experimentation.

The methods of treating individuals according to the invention include prophylactic treatment, therapeutic treatment, and curative treatment. Prophylactic treatment is treatment of

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an individual, using the methods of the invention, before any clinical sign of disease or infection is identified. Thus, prophylactic treatment is a preventative treatment and includes vaccination. Prophylactic treatment also includes treatment that is instituted after actual infection or disease inception, but before at least one clinical sign of the disease or infection is observed. Therapeutic treatment is treatment of an individual after at least one clinical sign of disease or infection is identified, or after an individual is known to (or is highly suspected of) having been exposed to a quantity of an agent that sufficient to cause disease or infection. Therapeutic treatment methods do not necessarily result in elimination of the disease or infection; however they do provide a clinically detectable improvement in at least one clinical sign of the disease or infection. Curative treatment methods result in complete elimination of the clinical signs of the disease or infection in the treated individual. Included in the curative treatment methods are those that result in complete removal of the causative agent of the infection or disease, whether it be a virus, bacterium, or host cell (such as a cancerous cell). Also included in curative treatment methods are those that cause complete remission of a disease, *i.e.*, complete elimination of all outward clinical signs of infection or disease, and repression of all detectable clinical manifestations of the infection or disease.

With respect to rabies vaccination, it is known that the virus can be treated both prophylactically (*e.g.*, by vaccination of dogs) and curatively (*e.g.*, by a series of injections to a human previously bitten by a rabid dog). Thus, a preferred embodiment of the present invention is a method of vaccinating an individual with a vaccine comprising the immunogenic composition of the invention. As discussed above, the immunogenic composition of the

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invention can comprise (or encode) multiple antigenic determinants. Therefore, a method of the invention can include multiple types of treatment for multiple types of diseases or infections. For example, a single method of treatment can comprise prophylactic treatment of polio, prophylactic and therapeutic treatment of rabies, and prophylactic treatment of influenza.

It will be apparent to those of ordinary skill in the art that various modifications and variations can be made in the construction of the molecules, and practice of the methods of the invention without departing from the scope or spirit of the invention. The invention will now be described in more detail with reference to specific examples of the invention, which are not intended to be, and should not be construed as, limiting the scope of the invention in any way.

EXAMPLES

For the Examples, the following materials and methods were used, unless otherwise specifically stated.

Mice. Female BALB/c (H-2^d BALB/C), 6 to 8 weeks of age, and Swiss (14 to 16 g) mice were purchased from "Centre d'Elevage et de Recherche" Janvier (Legenest St Isle, France).

Cells and lyssaviruses. BHK-21 cells used for the production and titration of lyssaviruses were grown in Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum (FBS) and 5% new born calf serum (Perrin, P., 1996, "Techniques for the preparation of rabies conjugates", *In* Laboratory techniques in rabies, Meslin, F-X., Kaplan, M., and Koprowski, H. Eds., (WHO Geneva):433-445). Neuroblastoma cells (Neuro-2a) used for transfection studies with plasmids were grown in MEM containing 8% FBS.

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The interleukin-2 (IL-2)-dependent cytotoxic T cell line (CTLL) was cultured as previously described (Perrin, P. *et al.*, 1988, "Interleukin-2 increases protection against experimental rabies", *Immunobiol.* **177**:199-209) in RPMI-1640 medium (Gibco : Flowbio, Courbevoie, France) containing 10% FBS, 1mM sodium pyruvate, 1mM non-essential amino acids, 5×10^{-5} M 2-mercaptoethanol, HEPES buffer (Flow Laboratories, Bethesda, MD, USA) and 5 to10 units (for 1 x 10^4 cells) of rat IL-2 (supernatant of splenocytes stimulated with concanavilin A: Con A). Cells were incubated at 37°C in a humidified atmosphere containing 7.5% CO_2.

Fixed PV-Paris/BHK-21, CVS rabies strains as well as rabies-related virus strains (EBL1b, EBL2b, LCMV, and Mok) were multiplied (passaged) in BHK-21 cells as previously described by Perrin, P., 1996, *supra*. The European bat lyssaviruses used were EBL1b (strain number 8916FRA) derived from a bat isolate from France and EBL2b (strain number 9007FIN; a gift from H. Bouhry) isolated from a human in Finland (Amengal, B. *et al.*, 1997, "Evolution of European bat lyssaviruses", *J. Gen. Virol.* **78**:2319-2328). The LCMV strain Arm/53b was kindly provided by Drs. M. Oldstone and M. McChesney (Scripps Clinic, La Jolla, CA).

Rabies virus antigens and vaccines. Inactivated and purified lyssaviruses (IPRV) were prepared as described by Perrin, P., 1996, *supra*. Virus was purified from inactivated (β-propiolactone) and clarified infected-cell supernatants by ultracentrifugation through a sucrose gradient. PV glycoprotein was solubilized from IPRV and purified (G PV) as previously described by Perrin, P., 1996, *supra* and Perrin, P., *et al.*, 1985, "Rabies immunosomes (subunit vaccine) structure and immunogenicity", *Vaccine*, 3:325-332.

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The two inactivated rabies virus used for comparative protection studies were prepared with two different strains: 1) PM as commercial vaccine for human use; (Pasteur Vaccines Marnes-la-Coquette France; Lot Y0047); 2) PV as a vaccine for laboratory use (IPRV).

Construction of plasmids expressing lyssavirus G genes. The region (amino acids 253-275) overlapping the only non-conformational epitope (VI) (Figure 1) was chosen for the construction of chimeric genes because it is presumably less structurally constrained than the two major antigenic sites II and III. The homogeneous and chimeric lyssavirus G genes (see Figure1) were introduced into the eukaryotic expression vector pCIneo (Promega), propagated and amplified in *E. coli* strain DH5α by standard molecular cloning protocols well known to the skilled artisan. Plasmids pGPV-PV and pGMok-PV were prepared as previously described (Bahloul, C., *et al.*, 1998, "DNA-based immunization for exploring the enlargement of immunological cross-reactivity against the lyssaviruses", *Vaccine* 16:417-425). Plasmids pGPV-Mok, pG-PVIII and pGEBL1-PV were obtained as follows.

For pGPV-Mok, the coding sequence of the site II part of G PV (amino acids 1-257) was amplified by RT-PCR using degenerated primers:

PV*Xba*I : 5' TTCTAGAGCCACCATGGTTCCTCAGGCTCTCCTG 3' (SEQ ID NO.:1)

PV*Bcl*I : 5' ATTGATCAACTGACCGGGAGGGC 3' (SEQ ID NO.:2)

The PCR product was inserted into the *Sma*I site of pUC19, then excised with *Bcl*I and *Eco*RI and ligated between the same sites in pGMok-Mok giving pGPV-Mok, containing an in-frame fusion of amino acids 1-257 from G PV with amino acids 258-503 from G Mok.

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The pG-PVIII gene, with an internal in-frame deletion between the end of the PV signal peptide and residue 253, was obtained by introducing a synthetic adaptor between the *Eco*RI and *Bcl*I restriction sites of the pGMok-PV plasmid. This PV adaptor, containing a single *Eco*RI site, was generated by annealing 200 picomoles of each primer in 250mM Tris-HCl pH 7.7. pG-PVIII was deposited on December 22, 1998 with the Collection Nationale de Cultures de Microorganismes (CNCM), Paris, France, and given Accession Number I-2115.

PVp1: 5' AATTCTAGAGCCGCCACCATGGTTCCTCAGGCTCTCCTGTT
TGTACCCCTTCTGGTTTTTCCATTGTGTTTTGGGAAGAATTCCC
CCCCCGGTCAGTT 3' (SEQ ID NO.: 3)

Pvp2: 5' GATCAACTGACCGGGGGGGAATTCTTCCCAAAACACAATG
GAAAAACCAGAAGGGGTACAAACAGGAGAGCCTGAGGAACCA
TGGTGGCGGCTCTAG 3' (SEQ ID NO.: 4)

To generate the pGEBL1-PV gene, a synthetic adaptor corresponding to amino acids 2-14 from EBL-1a (strain 8615POL), single *Bst*EII and *Eco*RI restriction sites were generated in the same way as that for pG-PVIII, by annealing:

EBL1p1: 5' AATTTCCCAATCTACACCATCCCGGATAAAATCGGACC
GTGGTCACCTATTCCG 3' (SEQ ID NO.: 5)

EBL1p2: 5' AATTCGGAATAGGTGACCACGGTCCGATTTTATCCGGG
ATGGTGTAGATTGGGA 3' (SEQ ID NO.: 6)

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This EBL1 adaptor was ligated in-frame into the *Eco*RI site of pG-PVIII. A fragment corresponding to amino acids 15-253 from EBL-1a (strain 8615POL) was then generated by RT-PCR of viral RNA using primers EBL1p3 and EBL1p4:

EBL1p3: 5' CCGTGGTCACCTATTGATATAAACCATCTCAGCTGCCCAA ACAACTTGATCGTGGAAGATGAG 3' (SEQ ID NO.: 7)

EBL1p4: 5' GGAATTCGAGCACCATTCTGGAGCTTC 3' (SEQ ID NO.: 8)

The RT/PCR product was digested with *Bst*EII and *Eco*RI and was ligated into the same sites introduced via the EBL1 adaptor, resulting in an in-frame fusion between the PV signal peptide, the EBL 1a site II part and the PV site III part. The identity of each construct was confirmed by automatic sequencing with dye terminator reaction on an ABI 377 sequencer (Perkin-Elmer). pEBL1-PV was deposited with the CNCM on December 22, 1998, and assigned the Accession Number I-2114.

Insertion of foreign B and CD8 cell epitopes in truncated or chimeric G genes. The previously reported truncated (pGPVIII) and chimeric genes (pGEBL1-PV) containing a unique *Eco*RI cloning site were used for the insertion of foreign B and CD8 cell epitopes. Lyssavirus G genes were introduced into the eukaryotic expression vector pCIneo (Promega), propagated and amplified in *Escherichia coli* strain DH5α by standard molecular cloning protocols.

B and CD8 cell epitopes were inserted into the *Eco*RI restriction site of the truncated or chimeric lyssavirus G genes in the hinge region (amino acids 253 to 275) of the molecule at position 253. The B cell epitope (named «B») corresponded to fragment C3 (amino acid 93 to 103: DNPASTTNKDK: SEQ ID NO.: 9) of the poliovirus VP1 protein. The CD8 cell epitope

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(named «CTL») corresponded to amino acids 119-127 (PQASGVYMG: SEQ ID NO.: 10) or amino acids 117-132 (ERPQASGVYMGNLTAQ: SEQ ID NO.: 11) of the lymphocyte choriomeningitis virus nucleoprotein. Plasmids p(B-CTL)2-GPVIII, pGEBL1-(B)-PV, pGEBL1-(CTL)-PV, pGEBL1-(B-CTL)-PV, pGEBL1-(B-CTL)$_2$-PV, and pGEBL1-(CTL-B)-PV were obtained as follows (see also Figure 1):

The p(B-CTL)2-GPVIII gene was generated by a two step cloning of a synthetic adapter in the unique *Eco*RI restriction site by annealing 200 picomoles of each primer:

«B-CTLp1»: 5' AATTCAGATAACCCGGCGTCGACCACTAACAAGGATAAGCTGTTCG CAGTGCCTCAGGCCTCTGGTGTGTATATGGGT 3' (SEQ ID NO.: 12)

«B-CTLp2»: 5' AATTACCCATATACACACCAGAGGCCTGAGGCACTGCGAACAGCTT ATCCTTGTTAGTGGTCGACGCCGGGTTATCTG 3' (SEQ ID NO.: 13).

For pGEBL1-(B)-PV, pGEBL1-(CTL)-PV, the synthetic adaptors used for the insertion were respectively:

B: «Bp3»: 5' AATTTGGATAACCCGGCGTCGACCACTAACAA 3' (SEQ ID NO.:14)

«Bp4»: 5' AATTCTTATCCTTGTTAGTGGTCGACGCCGGG 3' (SEQ ID NO.: 15)

CTL: «CTLp5»: 5' AATTTGGAGAGACCTCAGGCCTCTGGTGTGTATATGG GTAATCTTACGGCCCAG 3' (SEQ ID NO.: 16)

«CTLp6»: 5' AATTCTGGGAAGTAAGATTACCCATATACACACCAGAG GCCTGAGGTCTCTCCA 3' (SEQ ID NO.: 17).

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For pGEBL1-(B-CTL)-PV and pGEBL1-(CTL-B)-PV plasmid construction, pGEBL1-(B)-PV and pGEBL1-(CTL)-PV were used under the same conditions as above to insert CTL and B sequences, respectively, in the chimeric genes.

The identity of each construct was confirmed by automatic sequencing with dye terminator reaction on an ABI 377 sequencer (Perkin-Elmer).

Transient expression experiments. The ability of plasmids to induce transient expression of G related antigens was tested after transfection of Neuro 2a cells using the DOTAP cationic liposome-mediated method according to the manufacturer's instructions (Boehringer Mannheim). Each well of a cell culture microplate (Falcon) was inoculated with 3×10^4 cells (in MEM, 10% FBS) and incubated for 24 h at 37°C in a humidified atmosphere containing 7.5% CO_2. The plate was then washed with MEM without FBS and incubated as above for 1h. The cell supernatant was removed, and the wells were washed and filled with a total volume of 50 µl transfection solution, which contained 0.1 µg plasmid and 6 µl DOTAP ((N-(1-2,3,-dioleoyloxy) propyl)-N,N,N-trimethylammoniummethyl-sulfate) in sterile HEPES-buffered saline (150 mM NaCl, 20 mM HEPES) previously incubated at room temperature for 15 min. The plate was incubated for 5 h at 37°C in the presence of 7.5% CO_2. Two hundred µl MEM containing 2% FBS were added to each well and the plate was incubated for 24 to 140 h in the same conditions, before analysis of transient expression by indirect immunofluorescence.

The ability of plasmids to induce transient expression of G and foreign related antigens was tested after transfection of Neuro 2a cells using the FuGENE 6 transfection reagent according to the manufacturer's instructions (Boehringer Mannheim). Each well of a cell culture

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Labtek chamber Nunc (Life Technologies) was inoculated with 3 x 10^4 cells (in MEM, 10% FBS) and incubated for 24 h at 37°C in a humidified atmosphere containing 7.5% CO_2. The plates were then washed with MEM without FBS and wells were filled with 50 µl transfection solution: 0.1 µg plasmid, 3 µl of FuGENE 6 transfection reagent, and 47 µl of HEPES-buffered saline. The plate was incubated for 18 h at 37°C in the presence of 7.5% CO_2. Two hundred µl MEM containing 5% FBS were added to each well and the plate was incubated another 24 h under the same conditions before analysis of transient expression by indirect immunofluorescence.

Antibodies. Polyclonal antibodies (PAb) directed against PV and Mok G were obtained as described by Perrin, P., 1996, "Techniques for the preparation of rabies conjugates", *In* Laboratory techniques in rabies, Meslin, F-X., Kaplan, M., and Koprowski, H. Eds. (WHO Geneva):433-445, by rabbit immunization with purified virus glycoprotein. PAb against EBL-1b virus was obtained by mouse immunization with inactivated and purified virus.

Three monoclonal antibodies (MAb) directed against PV G were also used. PVE12 MAb (a MAb developed by M. Lafon *et al.*, 1985, "Use of a monoclonal antibody for quantitation of rabies vaccine glycoprotein by enzyme immunoassay", *J. Biol. Standard* 13:295-301) recognizes site II of native G. D1 MAb (IgG 1 isotype), produced in our laboratory, recognizes site III of native but not SDS-treated G. 6A1 MAb (a MAb reported in Lafay *et al.*, 1996, *J. Gen. Virol.* 77:339-346) recognizes SDS-denatured G protein and more precisely two peptides located downstream from site III, near the COOH-terminal part of the G ectodomain (amino acids 342-433 and 397-450).

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Immunofluorescence microscopy. Transient expression of G antigens in transfected cells was assessed with and without permeabilization (30 min incubation with 80% acetone on ice followed by air drying). Transfected cells were incubated for 1 h at 37°C with PAb or MAb. They were washed with PBS, and incubated for 1 h at 37°C with goat anti-rabbit or anti-mouse FITC-conjugated secondary antibody (Nordic Immunol. Labs, The Netherlands). Cells were washed, mounted in glycerol, and examined in a Leica inverted fluorescence microscope.

Two mouse monoclonal antibodies directed against PV G (D1 MAb IgG 1 isotype) and poliovirus (C3 MAb) were used for antigen staining by indirect immunofluorescence (IIF). D1 MAb recognizes the site III of native but not SDS-treated G and C3 MAb recognizes the 93-103 region of the PV-1 capsid VP1 protein. A rabbit neutralizing polyclonal antibody directed against the native poliovirus type 1 was also used.

Transient expression was assessed after 3% paraformaldehyde (Sigma) fixation (20 min incubation at room temperature) without permeabilization. Fixed cells were incubated for 1 h at room temperature with MAb. They were washed with phosphate-buffered saline (PBS), and incubated for 1 h at room temperature with goat anti-mouse or anti-rabbit FITC-conjugated secondary antibody (Nordic Immunol. Labs, The Netherlands). Cells were washed, mounted in Mowiol (Sigma) and examined in a Leica inverted fluorescence microscope.

Putative PEST sequence analysis. Polypeptide PEST sequences potentially involved in rapid degradation of protein were analyzed with the computer program PEST find developed according to Rogers, S. *et al.*,1986, "Amino acid sequences common to rapidly degraded proteins: the PEST hypothesis", *Science* 234:364-369.

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Injection of plasmids into mice. For immunological studies, BALB/c mice were anesthetized with pentobarbital (30 mg/kg) and 20-50 µg plasmid (diluted in PBS) was injected into each anterior tibialis muscle. This was more effective than injection via the quadriceps route.. Blood was collected for antibody assay of serum on various days by retro-orbital puncture.

For protection studies against LCMV, mice received 3.5 µg of cardiotoxin (Latoxan A.P., Les Ulis, France) in each leg four days before anaesthesia and immunization to degenerate the muscle.

Interleukin-2 release assay. Fourteen days after injection, spleens were removed from naive, or plasmid-injected BALB/c mice. Splenocytes (1 ml aliquots containing $6x10^6$) were stimulated with 0.5 µg lyssavirus antigen (*e.g.*, IPLV-PV and IPLV-EBL1) or 5 µg concanavilin A (Miles) in 24-well plates (Nuclon-Delta, Nunc) and cultured according to standard procedures in RPMI-1640 medium (Gibco) containing 10% FBS, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 5×10^{-5} M 2-mercaptoethanol, 10 mM HEPES buffer (Flow Laboratories). Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 7% CO_2. Under these conditions, cells producing IL-2 are mainly $CD4^+$ cells. IL-2 produced in supernatants of splenocyte cultures was titrated by bioassay using CTLL cells as previously described by Perrin, P. *et al.*, 1996, "The antigen-specific cell-mediated immune response in mice is suppressed by infection with pathogenic lyssaviruses", *Res. Virol.* 147:289-299. Cell proliferation was determined in triplicate, based on the uptake of ^{3}H-thymidine (New England Nuclear). IL-2 concentration was determined as units per ml (U/ml) using mouse recombinant IL-2 (Genzyme

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Corporation, Cambridge, MA, USA) as the reference. CTLL cells grew in the presence of mouse IL-2 and anti-IL-4 antibodies but not in the presence of IL-4 (up to 10 U/ml) and in the absence of IL-2. IL-2 was predominantly detected by this technique.

Antibody assays. For antibody assay of serum, blood was collected on various days by retro-orbital puncture. Rabies IgG were titrated by enzyme-linked immunosorbent assay (ELISA) using microplates coated with purified rabies glycoprotein. Titer corresponded to the reciprocal dilution of the serum sample giving the optical density equivalent to twice that given by serum (diluted 1/20) of PBS injected mice. Total anti-rabies IgG or IgG1, IgG2a and IgG3 isotypes were assayed by ELISA using microplates coated with IPRV as previously described (Perrin, P. *et al.*, 1986, "The influence of the type of immunosorbent on rabies antibody EIA; advantages of purified glycoprotein over whole virus", *J. Biol. Standard.* **14:**95) with rabbit anti-mouse IgG isotype sera as the secondary antibody (Nordic Immunol. Labs, The Netherlands) and a goat anti-rabbit IgG peroxidase conjugate (Nordic Immunol. Labs, The Netherlands) as the tertiary antibody.

Lyssavirus neutralizing antibodies were titrated by the rapid fluorescent focus inhibition test (RFFIT) (described by Smith, J. *et al.*, 1996, "A rapid fluorescent focus inhibition test (RFFIT) for determining virus-neutralizing antibody", *In* Laboratory techniques in rabies, Fourth edition (Eds Meslin, F-X; Kaplan, M and Koprowski, H) WHO, Geneva.:181-189) with the previously described modifications of Perrin, P. *et al.*, 1986, *J. Biol. Standard.* **14:**95. Infected cell supernatants (PV, CVS, and EBL2 viruses) and purified viruses (Mok and EBL1 viruses) were used. Anti- PV or CVS antibody titers are expressed in international units per ml (IU/ml)

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using the 2nd International Standard (Statens Seruminstitut, Copenhagen, Denmark) as the reference. For antibody titer determination against other lyssaviruses, the serum dilution causing 50% inhibition of the fluorescent focus rate was defined as having the same VNAb titer as for reference assayed against CVS.

Antibodies to PV-1 were assayed by ELISA as previously described using microplates coated with a synthetic peptide constituted by a trimer of amino acids 93-103 of VP1. Anti-LCM IgG production was also tested by ELISA.

Protection test. The protective activity of vaccines and plasmids was determined according to the NIH potency test. Dilutions of vaccine were injected intra-peritoneally (i.p.) into mice on days 0 and 7 whereas plasmids (40 µg) were injected into each anterior tibialis muscle on day 0 only. Mice were then intra-cerebrally (i.c.) challenged on day 21 with about 30 LD_{50} of various lyssaviruses (CVS, LCMV, EBL1b, or EBL2b). Animals were observed for 28 days or alternatively sacrificed at day 21 post-challenge and blood samples collected in order to control the virus clearance and the anti-LCMV IgG production.

Example 1: *Transient expression of lyssavirus G genes*

Plasmids containing homogeneous (pGPV-PV), truncated (pG-PVIII), and chimeric (pGEBL1-PV, pGMok-PV and pGPV-Mok) lyssavirus G genes were used to transfect Neuro 2a cells. Cell staining by indirect immunofluorescence is reported in Figure 2 and can be summarized as follows.

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After transfection with pGPV-PV, antigen was detected with PV PAb (Figure 2A), PV D1 MAb (Figure 2B), or PV E12 MAb (not shown), mostly at the cell membrane (similar results with non-permeabilized cells, data not shown) and very few detected with 6A1 MAb (Figure 2C). Cells transfected with pG-PVIII were round in shape and completely stained (both cytoplasm and membrane) with PV PAb (Figure 2D) or 6A1 MAb (Figure 2F), but not with PV D1 MAb (Figure 2E). Cells transfected with pGEBL1-PV were stained mostly at the cell membrane with PV PAb (Figure 2G), PV D1 MAb (Figure 2H), or EBL-1 PAb (Figure 2I). Cells transfected with pGMok-PV were stained (mainly at the membrane) with PV PAb (Figure 2J), PV D1 MAb (Figure 2K), Mok PAb (Figure 2L), and very few stained with 6A1 MAb (not shown). Cells transfected with pGPV-Mok were stained with PV PAb (Figure 2M) or Mok PAb and round in shape (Figure 2N).

Cell transfection distinguishes two types of G antigens: 1) stained principally at the membrane of cells normal in shape, in particular using neutralizing MAb directed to site II (PV E12) and III (PV D1); and 2) stained in both the cytoplasm and membrane of cells round in shape, in particular using MAb (6A1), which recognizes the denatured G molecule.

The kinetics of G protein expression was studied upon transfection of cells with three representative plasmids: pGPV-PV (homogenous), pGEBL1-PV (chimeric), and pG-PVIII (truncated). pGPV-PV produced G antigens in about 60% of cells when stained with PV PAb, whereas very few cells were stained with PV 6A1 MAb at any time point (Figure 3A). About 55% of cells transfected with pGEBL1-PV were stained with PV PAb and up to 15% with 6A1 MAb (Figure 3B) indicating that some G molecules were denatured. Ten to twenty percent of

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cells transfected with pG-PVIII were round in shape and stained with PV PAb whereas 5 to 10% were positive with 6A1 MAb, indicating that G molecules were denatured (Figure 3C).

Example 2: *Induction of IL-2-producing cells*

The ability of the plasmids, pGPV-PV, pG-PVIII, pGEBL1-PV, pGMok-PV and pGPV-Mok to induce IL-2-producing cells was assayed and the results are shown in Figure 4. Plasmids with the site III part of PV, whether unfused (pG-PVIII) or with any lyssavirus site II part (pGPV-PV, pGEBL1-PV and pGMok-PV), efficiently induced IL-2 producing cells (240 to 550 mU/ml). This was true even for pG-PVIII, which, however, had only low efficiency for both cell transfection (see above) and antibody induction (see below). For the chimeric plasmids EBL1-PV and Mok-PV, the T-cell response was greater after stimulation with inactivated and purified PV than with EBL-1b or Mok viruses. This was not due to the quality of the purified antigens because immunization of BALB/c mice with PV, EBL-1b, or Mok inactivated and purified virus followed by *in vitro* stimulation with the same antigen induced similar levels of IL-2 production (PV: 250 mU/ml, EBL-1b: 350 mU/ml and Mok: 400 mU/ml). In contrast, the plasmid pGPV-Mok induced only a weak Th cell response (IL-2 titer: 50 mU/ml), which was similarly produced *in vitro* after stimulation with either inactivated and purified PV or Mok virus.

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Example 3: *Serological assays*

The truncated pG-PVIII plasmid did not induce the production of rabies antibodies, when assayed by RFFIT and ELISA. However when IL-2 was injected together with pG-PVIII, and then alone 7 days later, antibodies were detected on day 21 only by ELISA (data not shown). Thus, the site III part was expressed *in vivo* and induced a weak production of non-neutralizing antibodies, which was boosted by exogenous IL-2.

In contrast, when the site III part of PV was linked with the homologous site II part, as in pGPV-PV, it displayed strong immunogenicity. A single injection of pGPV-PV plasmid into mice resulted in high levels of VNAb measured 27 days later against both the homologous PV and CVS viruses and the heterologous EBL-2b virus (Figure 5A). The antibody isotype induced was mainly IgG 2a, but a weak IgG 1 response was also observed (data not shown). However, the correlation between VNAb titers against PV was stronger with IgG 2a (r=0.974) than with IgG 1 titer (r=0.71), indicating that VNAb induced by DNA-based immunization were mainly IgG 2a. The VNAb titer against the homologous PV and CVS viruses increased when mice received a booster injection on day 30 and their sera were checked at day 40, but not the VNAb titers against the heterologous EBL-2b virus which remained unchanged (Figure 5A). Likewise, under these conditions, a relationship between VNAb level induced by pGPV-PV and the protection of mice against an i.c. challenge with CVS was seen: all animals with a VNAb titer (on day 20) above 1.5 IU/ml survived the challenge on day 21 (not shown). In contrast, no significant amount of VNAb against EBL-1b was produced after a single injection or after a boost.

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In view of these results and a previous observation that the chimeric plasmid pGMok-PV induced VNAb against both PV and Mok viruses, the capability of the site III part of PV to carry the heterologous EBL1 site II part was investigated. A single injection of the chimeric plasmid pGEBL1-PV similarly induced VNAb against both PV and EBL-1b viruses (Figure 5B). Fourteen days after injection, titers were 2 IU/ml and they increased to 17 IU/ml after 80 days. The level of VNAb production against EBL-1b was always higher than that against PV, but the difference was not significant.

Taken together, these results clearly demonstrate that chimeric G genes encoding the site III part of PV and the site II part of G of other lyssaviruses such as EBL-1b or Mok are very potent inducers of VNAb against both parental viruses. In contrast, the symmetric pGPV-Mok construct did not induce VNAb against either PV or Mok viruses (not shown).

Example 4: *Protection assays against European lyssaviruses*

The ability of both the homogenous pGPV-PV and the chimeric pGEBL1-PV plasmid to induce protection against an i.c. challenge with viruses representing lyssavirus genotypes involved in the transmission of encephalomyelitis in Europe (CVS for GT1, EBL1b for GT5, and EBL2b for GT6) was tested. Their efficiency was compared with that of a commercially available vaccine (PM strain: GT1) and a laboratory preparation (PV strain: GT1), and the results are presented in Figure 6.

The plasmid backbone (pCIneo) did not induce protection against any virus (Figure 6d, e, and f). In contrast, pGPV-PV protected 70% of BALB/c (and 85% of Swiss) mice against CVS

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(Figure 6d), 30% against EBL1b (Figure 6e), and 72% against EBL2b (Figure 6f). In the same conditions, pGEBL1-PV protected 60% of BALB/c mice against CVS (Figure 6d), 75% against EBL1b (Figure 6e) and 80% against EBL2b (Figure 6f). Thus, if immunization with any of the two plasmids showed no significant difference in the protection against CVS (GT1) and EBL2b (GT6), the chimeric pGEBL1-PV was far more efficient against EBL1b (GT5) and is clearly the best candidate for protection with DNA-based immunization against the three European lyssavirus genotypes.

Concerning the protection induced by inactivated cell culture vaccines using the PM and PV strains against the same challenges, a human dose of PM vaccine diluted 1/10th protected 80% of mice against CVS (Figure 6a), 36% against EBL1b (Figure 6b), and 80% against EBL2b (Figure 6c). Under the same conditions, 2 µg of PV IPRV protected 100% of mice against both CVS (Figure 6a) and EBL1b (Figure 6b) and 85% against EBL2b (Figure 6c). For a vaccine dose that protected 80 to 85% of the animals against EBL2b, the PV strain protected 100% and PM strain only 36% against EBL1b. Thus, the PM strain is less effective than the PV strain against EBL1b.

Example 5: *Transient expression of lyssavirus G genes*

Plasmids containing the foreign antigen encoding sequences associated with the truncated (pG-PVIII) or chimeric (pGEBL1-PV) lyssavirus G genes were tested for their ability to transiently transfect Neuro 2a cells. Except for pG(B-CTL)$_2$-PVIII, which induced an IIF staining (in the cytoplasm) only after permeabilization, all plasmids induced the expression of

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polio- and lyssaviruses related antigens at the cell membrane of non-permeabilized cells, as previously reported for the same plasmids without foreign epitopes.

For illustration, transfection results obtained with pGEBL1-(B-CTL)$_2$-PV are reported in Figure 7. Both rabies virus G part recognized by PV D1 MAb (Figure 7A) and poliovirus insert recognized either by the C3 MAb (Figure 7B) or the anti poliovirus type 1 PAb (Figure 7C) were evidenced, whereas no staining was observed with the same MAb and PAb on PCIneo transfected cells (Figure 7D). This clearly indicates that, except for pG(B-CTL)$_2$-PVIII, the chimeric pGEBL1-PV glycoprotein allowed the expression of the poliovirus B cell epitope alone or in association with the LCMV CTL cell epitope at the cell surface membrane under a native form whereas the expression of lyssavirus G was maintained.

Example 6: *Immunogenicity of foreign epitopes carried by the truncated glycoprotein*

The truncated pGPVIII gene was used to carry and expressed C3 VP1 B cell and LCMV CD8$^+$ CTL epitopes in mice after DNA-based immunization.

While the pGPVIII gene induced IL-2 producing cells that can be stimulated *in vitro* by IPRV 21 days after injection, no production was observed after 14 days (Figure 8A). However, a significant production was observed with pG(B-CTL)$_2$-GPVIII after 14 days (Figure 8A). This indicates that the fusion of foreign epitopes with GPVIII significantly enhances the production of Th cells directed to site III part of rabies G.

Although pGPVIII induced no anti-rabies antibody in the absence of exogenous IL-2 (Figure 9), the kinetic study of antibody induced by pG(B-CTL)$_2$-GPVIII showed that significant

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antibody production occurred against both rabies G and poliovirus peptide (Figure 9). This also indicates that the fusion of foreign epitopes with GPVIII significantly enhances the production of antibody directed to site III part of rabies PV G. Moreover, the truncated GPVIII was able to carry and to allow the expression of poliovirus B cell epitope *in vivo* with antibody production.

Production of antibodies induced by p(B-CTL)$_2$-GPVIII against a poliovirus peptide was also tested after priming with either pGPVIII or p(B-CTL)$_2$-GPVIII itself (Figure 10). When p(B-CTL)$_2$-GPVIII was injected without priming and controlled 13 days (PBS / p(B-CTL)2-GPVIII -D26-) or 39 days after (p(B-CTL)2-GPVIII -D0-), anti-peptide antibody titer was 65 and 80, respectively. However, if a priming was performed with pGPVIII or p(B-CTL)$_2$-GPVIII, the titer was 200 and 600, respectively. This clearly demonstrates that the two types of priming enhanced antibody production against a poliovirus peptide.

<u>Example 7</u>: *Immunogenicity of foreign epitopes carried by the chimeric glycoprotein*

Immunogenicity of both B and CD8 T cell epitopes and the consequence of their insertion on the immunogenicity of the chimeric glycoprotein were analyzed according to humoral and cell mediated immune responses after DNA-based immunization. When epitopes were inserted in the chimeric pGEBL1-PV plasmid, the induction of IL-2-producing cells able to be stimulated by IPLV depended on inserted epitopes. Two types of results were obtained, and are shown in Figure 8B: i) IL-2 production induced by pGEBL1-(B-CTL)$_2$-PV, pGEBL1-(CTL-B)-PV, and pGEBL1-(CTL)-PV was similar to that induced by pGEBL1-PV; and ii) IL-2 production induced by pGEBL1-(B-CTL)-PV (data not shown) and pGEBL1-(B)-PV was inhibited. Consequently,

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the chimeric G EBL1-PV can carry foreign B and CD8 T cell epitopes without negative effects on its ability to induce IL-2-producing cells. However, concerning the B cell epitope, a position effect was observed since its insertion immediately behind the EBL1 sequence was deleterious for the induction of T helper cells stimulated by lyssavirus G. However, this phenomenon was not evidenced with pGEBL1-(B-CTL)$_2$-PV.

Insertion of foreign epitopes in pGEBL1-PV was also studied for its consequence on the induction of antibodies against poliovirus peptide and VNAb against both PV and EBL1 lyssaviruses. Table 1 shows antibody production induced by the chimeric pGEBL1-PV plasmid carrying various foreign epitopes. BALB/c mice (three for each plasmid) were injected with 50 µg of various plasmids in each tibialis muscle. Sera were assayed on day 21 for rabies or EBL1 virus neutralizing antibody by the RFFIT method (titer expressed in IU/ml) and for poliovirus anti-peptide antibody by ELISA. Results are expressed as the mean titer, and standard deviation are reported in brackets.

The four plasmids containing the B cell epitope (pGEBL1-(B)-PV, pGEBL1-(CTL-B)-PV, pGEBL1-(B-CTL)$_2$-PV, and pGEBL1-(B-CTL)-PV) induced antibody against the poliovirus peptide. However, when the B cell epitope followed EBL1 sequence (pGEBL1-(B-CTL)-PV and pGEBL1-(B)-PV), antibody production was weaker than when the B epitope was separated by the CD8 cell epitope (pGEBL1-(CTL-B)-PV). The results for pGEBL1-(B-CTL)$_2$-PV were intermediary. The insertion of foreign epitopes induced a decrease of anti-lyssavirus VNAb production but was maintained at a high level when animals were injected with pGEBL1-(CTL)-

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PV or pGEBL1-(CTL-B)-PV. However, when the B cell epitope was inserted immediately behind the EBL1 sequence, not as great of an anti-lyssavirus VNAb production was observed.

In summary, the chimeric G EBL1-PV can carry and allow the *in vivo* expression of both poliovirus and lyssavirus neutralizing B cell epitopes but the presence of the poliovirus B cell epitope immediately behind the EBL1 sequence (excepted for $(B\text{-}CTL)_2$ insertion) is deleterious for the immunogenicity of both the site II and site III part of the chimeric glycoprotein. On the other hand, when the foreign epitopes are fused with the truncated G PVIII, both T helper and non-neutralizing antibody production can be induced.

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TABLE 1. Antibody production induced by the chimeric pGEBL1-PV plasmid carrying various combinations of B and CD8$^+$ T cell foreign epitopes.

Plasmid injected	Neutralizing antibody (IU/ml) against: Rabies virus	European Bat Lyssavirus 1	Poliovirus anti-peptide antibody (Reciprocal dilution)
pGEBL1-(B-CTL)$_2$-PV	0.9 (0.05)	1.1 (0.07)	1100 (200)
pGEBL1-(CTL-B)-PV	1.8 (0.2)	8.0 (1.0)	1510 (490)
pGEBL1-(B-CTL)-PV	0.06 (0.06)	0.6 (0.07)	810 (210)
pGEBL1-(CTL)-PV	2.6 (0.2)	5.2 (0.2)	0 (0)
pGEBL1-(B)-PV	0.1 (0.01)	0.21 (0.09)	355 (45)
pGEBL1-PV	5.9 (2.1)	21.9 (1.8)	0 (0)
pClneo	0 (0)	0 (0)	0 (0)

TABLE 1

Example 8: *Protection against a lethal dose of LCMV*

As the CD8 T cell epitope is involved in the induction of protection against LCMV, the truncated and chimeric G carrying the LCMV $CD8^+$ T cell epitope were tested for their protective activity. Table 2 shows the protection induced by the chimeric pGEBL1-PV plasmid carrying the LCMV $CD8^+$ epitopes. BALB/c mice (three for each plasmid) were injected with 40 μg of various plasmids and i.c. challenged on day 21. Percentage of surviving animals is reported in brackets.

The truncated $p(B\text{-}CTL)_2$-GPVIII induced only a partial protection. When the B and CD8 T cell epitopes were inserted into the chimeric GEBL1-PV, a significant protection was observed with pGEBL1-(CTL-B)-PV against a lethal challenge with LCMV (70% of mice survived). Under these conditions, the surviving animals completely eliminated the virus when controlled by RT-PCR 21 days post-infection (data not shown). This indicates that the chimeric G is a very potent carrier of a protective CD8 cell epitope. However, as for anti-lyssavirus and poliovirus immune responses, the insertion of the poliovirus B cell epitope immediately behind the EBL1 sequence induced an inhibition of the protective activity of the following CD8 cell epitope.

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TABLE 2. Protection induced by the truncated pGPVIII and the chimeric pGEBL1-PV plasmid encoding the LCMV CD8+ epitope.

Plasmid injected	Survival animals (%)
pCIneo	0/10 (0)
$p(B\text{-}CTL)_2$-GPVIII	2/5 (40)
pGEBL1-(B-CTL)-PV	0/10 (0)
pGEBL1-(CTL-B)-PV	7/10 (70)

TABLE 2

Example 9: *Putative PEST sequence*

Since a deleterious position effect was clearly observed for the position of the insertion of the B cell epitope before or after the CTL cell epitope, the presence of putative PEST sequences in the different plasmids was analyzed (Figure 1D). Only two plasmids (pGEBL1-(B)-PV and pGEBL1-(B-CTL)$_2$-PV) contained putative PEST sequences due to the junction of the end of EBL1 and the B cell epitope sequences. However, the substitution of the serine (S) in pGEBL1-(B-CTL)$_2$-PV by a leucine (L) in pGEBL1-(B)-PV reduced the PEST value from +8.38 to +4.20.

Example 10: *Dog immunization and challenge*

Beagle dogs ranging in age from 4 to 8 years were assigned in four experimental groups (Table 3). They were kept in isolated cages and fed with commercial food (400 g each day). They were injected intramuscularly in the thigh with either with 100 µg of plasmid (group A, B, and C) or PBS (group D). Plasmid was injected in one site on day 0, 21, 42, and 175 (group A) or on day 0 and 175 (group C). It was also injected in three sites (3 x 33 µg) on day 0, 21, 42, and 175 (group B). Blood samples were collected (from veinpuncture) on day 0, 28, 49, 70, 120, 175, and 189.

Lyssavirus neutralizing antibodies were titrated by the rapid fluorescent focus inhibition test (RFFIT) with the previously described modifications using PV, CVS, or FWR viruses. Anti-PV, CVS, or FWR neutralizing antibody titers are expressed in international units per ml (IU/ml) using the 2nd International Standard (Statens Seruminstitut, Copenhagen, Denmark) as the reference or as the reciprocal serum dilution that inhibit 50% of fluorescent focus. Under these

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conditions, a titer of 40, 70, or 60 (reciprocal serum dilution) was equivalent to 0.5 IU/ml against PV, CVS or FWR respectively.

As shown in Figure 11, a virus neutralizing antibody (VNAb) can be induced by the plasmid containing the gene encoding the G protein groups (A, B, and C), whereas no antibody was detected in PBS injected animals (group D) whatever the injection protocol. Heightened levels of VNAb were obtained after one injection and a boost on day 21 (group A) or on day 175 (group C). As the seroconversion occurs for 0.5 IU/ml, it was concluded that all animals serconverted.

Moreover, the results presented in Figure 12 show that all plasmid-injected (vaccinated) dogs were protected against a rabies virus challenge. This is in contrast to the two unvaccinated animals (dogs 11 and 12) that were challenged, but did not survive.

These results are representative and the dosage can be extrapolated to other rabies-susceptible animals, including humans.

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Table 3: Dog characteristics and injection protocol

Group	Dog number	Sex*	Age (years)	Injection Product injected	Injection Site Number	Injection Day
A	1	M	8	Plasmid	1	0, 21, 42 and 175
	2	F	6	Plasmid	1	0, 21, 42 and 175
	3	F	6	Plasmid	1	0, 21, 42 and 175
B	4	F	6	Plasmid	3	0, 21, 42 and 175
	5	M	6	Plasmid	3	0, 21, 42 and 175
	6	F	8	Plasmid	3	0, 21, 42 and 175
C	7	M	4	Plasmid	1	0 and 175
	8	F	7	Plasmid	1	0 and 175
	9	F	4	Plasmid	1	0 and 175
D	10**	M	4	PBS	1	0, 21, 42 and 175
	11	M	4	PBS	1	0, 21, 42 and 175
	12	M	4	PBS	1	0, 21, 42 and 175

* M: male; F: female

** Discarded on day 160 (sick)

TABLE 3

Example 11: *pG-PVIII as a carrier for Plasmodium proteins*

Malaria during a woman's first pregnancy results in a high rate of fetal and neonatal death. The decreasing susceptibility during subsequent pregnancies correlates with acquisition of antibodies that block binding of infected red blood cells to chondroitin sulfate A (CSA), a receptor for parasites in the placenta. The inventors have identified a domain within a particular *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) that binds CSA (Buffet *et al.*, 1999, "*P. falciparum* domain mediating adhesion to chondroitin sulfate A: A receptor for human placental infection", *PNAS*, 96:12743-48). More specifically, a *var* gene, which is a *Plasmodium*-derived protein that is expressed in CSA-binding parasitized red blood cells (PRBCs), was cloned. The gene encodes a protein that has eight receptor-like domains. The inventors and their colleagues successfully demonstrated that the Duffy-binding-like (DBL) domain called DBL-3 binds CSA and displays the same binding specificity as PRBCs.

The DBL-3 domain contains a number of cysteines, which seem to be important for the correct folding of the CSA binding region. This folding can, most likely, not be achieved in bacterial expression systems, but can certainly be achieved using a eukaryotic expression system.

A chimeric expression plasmid (DBL-3/PVIII) having 1) a nucleic acid sequence encoding the DBL-3 domain and 2) a nucleic acid sequence encoding the PVIII site was prepared and transfected into N_2A cells. After transfection, washing, and outgrowth of transfected cells in microtiter plate wells, the cells were fixed with 80% acetone for 10 minutes on ice, then subjected to indirect immunofluorescence to determine whether the DBL-3 and/or PVIII epitopes were expressed, and if so, where the encoded epitopes were located within the cells.

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Figure 13 shows that both the DBL-3 domain and the PVIII site were efficiently expressed at the membrane of transfected cells. This result has a great number of implications, not the least of which is that expression of the heterologous *Plasmodium* DBL-3 epitope on the surface of eukaryotic cells using a chimeric nucleic acid carrier molecule of the invention can be accomplished. For example, expression of the DBL-3 epitope from a carrier molecule of the invention can be used to induce immunity that protects a pregnant woman and her fetus from the harmful and potentially deadly effects of malaria.

Example 12: *Protection against malaria*

Because protective antibodies present after pregnancy block binding of parasites from different parts of the world to CSA, pregnant women and their fetuses can be protected via cross-reactivity induced by exposure to DBL-3. Thus, vaccines containing or expressing the DBL-3 domain can be used to immunize women, including pregnant women, especially those women in tropical regions of Africa, Asia, and South and Central America.

Thus, the present invention provides an excellent means to induce antibodies capable of interfering with the CSA binding of infected erythrocytes, thereby protecting women and their fetuses from the harm caused by malaria.

A DNA vaccine is administered to women who are pregnant or who are planning to become pregnant. The DNA vaccine comprises a chimeric nucleic acid carrier molecule, in which an expression vector having a nucleic acid sequence encoding the DBL-3 domain is fused to a nucleic acid sequence encoding the rabies PVIII site. Fusion of the DBL-3 encoding

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sequences to the rabies glycoprotein PVIII sequences, and expression in the recipient woman, promotes efficient induction of an immune response in the woman against the encoded DBL-3 polypeptide sequence, and protects the woman and her fetus from the harmful effects of malaria.

The invention has been described in detail above with reference to preferred embodiments. However, it will be understood by the ordinary artisan that various modifications and variations can be made in the practice of the present invention without departing from the scope or spirit of the invention. All references cited herein are hereby incorporated by reference in their entirety.

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ABSTRACT OF THE DISCLOSURE

The present invention provides chimeric nucleic acids, preferably contained on an expression vector, that encode chimeric immunogenic polypeptides. The nucleic acids encode at least site III of a lyssavirus glycoprotein, which has been found to improve the immunogenicity of lyssavirus epitopes for protection from rabies. The chimeric nucleic acids and proteins can also contain antigenic determinants for epitopes other than those of lyssavirus. Thus, the invention provides chimeric nucleic acids and polypeptides that elicit a strong immune response to multiple antigens. Use of the methods of the present invention permits DNA vaccination without the need to supply multiple antigens on separate DNA molecules.

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1-57. (CANCELED)

58. (NEW) A polynucleotide comprising:

a) a polynucleotide encoding a site III polypeptide sequence of a lyssavirus glycoprotein from a genotype GT1 strain, wherein the polynucleotide does not encode an entire lyssavirus glycoprotein;

b) a polynucleotide encoding a site II polypeptide sequence of a lyssavirus glycoprotein from genotype GT5;

c) a polynucleotide encoding a transmembrane domain of a transmembrane protein; and

d) a polynucleotide encoding a cytoplasmic domain of a transmembrane protein.

59. (NEW) The polynucleotide of any one of claims 58, wherein said polynucleotide further comprises a heterologous polynucleotide encoding a peptide, polypeptide, or protein other than a lyssavirus glycoprotein or a peptide or polypeptide fragment of a lyssavirus glycoprotein.

60. (NEW) The polynucleotide of claim 59, wherein the heterologous polynucleotide encodes a B cell epitope, or a CD8 cell epitope or both.

61. (NEW) A polypeptide encoded by the polynucleotide of claim 59.

62. (NEW) An immunogenic composition comprising the polypeptide of claim 61.

63. (NEW) The immunogenic composition of claim 62, wherein said composition induces humoral and cellular immunity.

64. (NEW) The immunogenic composition of claim 62, wherein said immunogenic composition induces a protective immune response.

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Chimeric Lyssavirus Glycoproteins with Increased Immunological Potential

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The rabies virus glycoprotein molecule (G) can be divided into two parts separated by a flexible hinge: the NH_2 half (site II part) containing antigenic site II up to the linear region (amino acids [aa] 253 to 275 encompassing epitope VI [aa 264]) and the COOH half (site III part) containing antigenic site III and the transmembrane and cytoplasmic domains. The structural and immunological roles of each part were investigated by cell transfection and mouse DNA-based immunization with homogeneous and chimeric G genes formed by fusion of the site II part of one genotype (GT) with the site III part of the same or another GT. Various site II-site III combinations between G genes of PV (Pasteur virus strain) rabies (GT1), Mokola (GT3), and EBL1 (European bat lyssavirus 1 [GT5]) viruses were tested. Plasmids pGPV-PV, pGMok-Mok, pGMok-PV, and pGEBL1-PV induced transient expression of correctly transported and folded antigens in neuroblastoma cells and virus-neutralizing antibodies against parental viruses in mice, whereas, pG-PVIII (site III part only) and pGPV-Mok did not. The site III part of PV (GT1) was a strong inducer of T helper cells and was very effective at presenting the site II part of various GTs. Both parts are required for correct folding and transport of chimeric G proteins which have a strong potential value for immunological studies and development of multivalent vaccines. Chimeric plasmid pGEBL1-PV broadens the spectrum of protection against European lyssavirus genotypes (GT1, GT5, and GT6).

Rabies is a fatal form of encephalomyelitis caused by viruses of the *Lyssavirus* genus of the *Rhabdoviridae* family. On the basis of nucleotide sequence comparison and phylogenetic analysis, the *Lyssavirus* genus has been divided into six genotypes (GTs) (7). GT1 includes the classical rabies viruses and vaccine strains, whereas GT2 to GT6 correspond to rabies-related viruses, including Lagos bat virus (GT2), Mokola virus (Mok) [GT3], Duvenhage virus (GT4), European bat lyssavirus 1 (EBL1 [GT5]), and EBL2 (GT6). A new lyssavirus that may belong to a new genotype (GT7) has recently been reported in Australia (16). Based on antigenicity, the *Lyssavirus* genus was first divided into four serotypes (34) and was recently divided into two principal groups according to the cross-reactivity of virus-neutralizing antibodies (VNAbs) (group 1, GT1, GT4, GT5, GT6, and GT7; group 2, GT2 and GT3) (4). Viruses of group 2 are not pathogenic when injected peripherally in mice (29), and concerning amino acids that play a key role in pathogenicity, their glycoprotein is similar to that of the avirulent GT1 viruses (3, 8).

Currently available vaccines mostly belong to GT1, against which they give protection (23). However, the protection against GT4 to GT6 depends on the vaccine strain. (1, 11, 19). Concerning the protection against the EBLs (GT5 and GT6), the isolation of which has become more frequent in recent years, the rabies vaccine PM (Pitman-Moore) strain induces weaker protection against EBL1 than the PV (Pasteur virus) strain, and few data are reported for EBL2 (11, 19). It is therefore important to study ways of increasing the level of protection against these viruses or broadening the spectrum against rabies-related viruses.

Rabies virus glycoprotein molecule (G) is composed of a cytoplasmic domain, a transmembrane domain, and an ectodomain, exposed as trimers at the virus surface (9, 13). The ectodomain is involved in the induction of both VNAb production and protection after pre- and postexposure vaccination (32, 41). Therefore, much attention has been focused on G in the development of rabies subunit vaccines. It is generally thought that the G ectodomain has two major antigenic sites recognized by about 72.5% (site II) and 24% (site III) of neutralizing monoclonal antibodies (MAbs), respectively, one minor site (site a), and several epitopes recognized by single MAbs (I, amino acid [aa] 231; V, aa 294; and VI, aa 264) (5, 10, 18, 21). Site II is conformational and discontinuous (aa 34 to 42 and aa 198 to 200 associated by disulfide bridges), whereas site III is conformational and continuous (aa 330 to 338). Lysine 330 and arginine 333 in site III play a key role in neurovirulence and may be involved in the recognition of neuronal receptors (8, 40). Sites II and III seem to be close to one another and are exposed at the surface of the protein (12). However, at low pH, the G molecule takes on a fusion-inactive conformation in which site II is not accessible to MAbs, whereas sites a and III remain more or less exposed (14, 15). Moreover, several regions distributed along the ectodomain are involved in the induction of T helper (Th) cells (24, 42). Based on these structural and immunological properties, we have suggested that the G molecule may consist of two immunologically active parts, each potentially able to induce both VNAb and Th cells (4): the NH_2-terminal half containing antigenic site II (the site II part) and the COOH-terminal half containing site III and the transmembrane and cytoplasmic domains (the site III part).

In this study, we used in vitro transfection of neuroblastoma cells and DNA-based immunization to study the relative immunological importance of the site II and III parts of lyssavirus glycoproteins. This includes humoral, cell-mediated immune responses and protective activity. We assessed the immunogenic autonomy of each part by using homogeneous and chi-

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meric G genes formed by fusion of the site II part of one GT with the site III part of the same or another GT. The site III part of G of the PV strain (GT1) was the most effective at presenting in vivo the site II parts of various lyssaviruses: Mok (GT3) and EBL1b (GT5). However, the site II part seemed to be required for correct folding and transport of the site III part, as well as for potent immunological responses. We also demonstrated that the EBL1-PV chimeric plasmid gave protection against the challenge virus standard (CVS), EBL1b, and EBL2b, which represent the European lyssavirus GTs (GT1, GT5, and GT6, respectively). These findings show that the site II part of rabies virus G can be exchanged with other lyssavirus glycoproteins and illustrate the potential value of chimeric genes for immunological studies and multivalent vaccine development.

MATERIALS AND METHODS

Mice. Female BALB/c (6 to 8 weeks of age) and Swiss (body weight, 14 to 16 g) mice were purchased from "Centre d'Elevage et de Recherche" Janvier (Legenest St. Isle, France).

Cells and lyssaviruses. BHK-21 cells used for the production and titration of lyssaviruses were grown in Eagle's minimal essential medium (MEM) containing 5% fetal bovine serum (FBS) and 5% newborn calf serum (28). Neuroblastoma cells (Neuro-2a) used for transfection studies with plasmids were grown in MEM containing 8% FBS.

The interleukin-2 (IL-2)-dependent cytotoxic T-lymphocyte line (CTLL) was cultured as previously described (30) in RPMI 1640 medium (Gibco-Flowbio, Courbevoie, France) containing 10% FBS, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, HEPES buffer (Flow Laboratories, Bethesda, Md.), and 5 to 10 U (for 10^4 cells) of rat IL-2 (supernatant of splenocytes stimulated with concanavalin A [ConA]). Cells were incubated at 37°C in a humidified atmosphere containing 7.5% CO_2.

Fixed PV-Paris/BHK-21, CVS rabies strains as well as rabies-related virus strains (EBL1b, EBL2b, and Mok) were multiplied in BHK-21 cells as previously described (28). The EBLs used were EBL1b (strain 8916FRA), derived from a bat isolate from France, and EBL2b (strain 9007FIN: a gift from H. Bouhry), isolated from a human in Finland (1).

Rabies virus antigens and vaccines. Inactivated and purified lyssaviruses (IPRV) were prepared as described elsewhere (28). Virus was purified from inactivated (β-propiolactone) and clarified infected-cell supernatants by ultracentrifugation through a sucrose gradient. PV glycoprotein was solubilized from IPRV and purified (G PV) as previously described (28, 32).

The two inactivated rabies viruses used for comparative protection studies were prepared with two different strains: (i) PM as a commercial vaccine for human use (Pasteur Vaccins, Marnes-la-Coquette, France: lot Y0047) and (ii) PV as a vaccine for laboratory use (IPRV).

Construction of plasmids expressing lyssavirus G genes. The region (aa 253 to 275) overlapping the only nonconformational epitope (VI) (Fig. 1) was chosen for the construction of chimeric genes because it is presumably less structurally constrained than the two major antigenic sites, II and III (5, 10). The homogeneous and chimeric lyssavirus G genes (Fig. 1) were introduced into the eukaryotic expression vector pCIneo (Promega), propagated and amplified in *Escherichia coli* strain DH5α by standard molecular cloning protocols (25). Plasmids pGPV-PV and pGMok-PV were prepared as previously described (4). Plasmids pGPV-Mok, pG-PVIII, and pGEBL1-PV were obtained as follows.

For pGPV-Mok, the coding sequence of the site II part of G PV (aa 1 to 257) was amplified by reverse transcription-PCR (RT-PCR) by using the degenerated primers PV*Xba*I (5' TTCTAGAGCCACCATGGTTCCTCAGGCTCTCCTG 3') and PV*Bcl*I (5' ATTGATCAACTGACCGGGAGGGC 3'). The PCR product was inserted into the *Sma*I site of pUC19 and then excised with *Bcl*I and *Eco*RI and ligated between the same sites in pGMok-Mok, giving pGPV-Mok, which contains an in-frame fusion of aa 1 to 257 from G PV with aa 258 to 503 from G Mok.

The pG-PVIII gene, with an internal in-frame deletion between the end of the PV signal peptide and residue 253, was obtained by introducing a synthetic adapter between the *Eco*RI and *Bcl*I restriction sites of the pGMok-PV plasmid. This PV adapter, containing a single *Eco*RI site, was generated by annealing 200 pmol of each of the following primers in 250 mM Tris-HCl (pH 7.7): PVp1 (5'AATTCTAGAGCCGCCACCATGGTTCCTCAGGCTCTCCTGTTTGTA CCCCTTCTGGTTTTTCCATTGTGTTTTGGGAAGAATTCCCCCCCCGG TCAGTT 3') and Pvp2 (5'GATCAACTGACCGGGGGGGGAATTCTTCCC AAAACACAATGGAAAAACCAGAAGGGGTACAAACAGGAGAGCCTG AGGAACCATGGTGGCGGCTCTAG 3').

To generate the pGEBL1-PV gene, a synthetic adapter corresponding to aa 2 to 14 from EBL1a (strain 8615POL) (1) and single *Bst*EII and *Eco*RI restriction sites was generated in the same way as that for pG-PVIII by annealing with primers EBL1p1 (5'AATTTCCCAATCTACACCATCCCCGGATAAAATCGG ACCGTGGTCACCTATTCCG 3') and EBL1p2 (5'AATTCGGAATAGGT GACCACGGTCCGATTTTATCCGGGATGGTGTAGATTGGGA3'). This EBL1 adapter was ligated in frame into the *Eco*RI site of pG-PVIII. A fragment corresponding to aa 15 to 253 from EBL1a (strain 8615POL) was then generated by RT-PCR of viral RNA by using primers EBL1p3 (5'CCGTGGTCACCTAT TGATATAAACCATCTCAGCTGCCCAAACAACTTGATCGTGGAAGAT GAG3') and EBL1p4 (5'GGAATTCGAGCACCATTCTGGAGCTTC 3'. The RT-PCR product was digested with *Bst*EII and *Eco*RI and was ligated into the same sites introduced via the EBL1 adapter, resulting in an in-frame fusion between the PV signal peptide, the EBL1a site II part, and the PV site III part.

The identity of each construct was confirmed by automatic sequencing with dye terminator reaction on an ABI 377 sequencer (Perkin-Elmer).

Transient expression experiments. The ability of plasmids to induce transient expression of G-related antigens was tested after transfection of Neuro-2a cells by using the DOTAP [(*N*-(1-2,3,-dioleoyloxy)propyl)-*N,N,N*-trimethylammoniummethyl-sulfate] cationic liposome-mediated method according to the manufacturer's (Boehringer Mannheim) instructions. Each well of a cell culture microplate (Falcon) was inoculated with 3×10^4 cells (in MEM–10% FBS) and incubated for 24 h at 37°C in a humidified atmosphere containing 7.5% CO_2. The plate was then washed with MEM without FBS and incubated as described above for 1 h. The cell supernatant was removed, and the wells were washed and filled with 50 μl of transfection solution, containing 0.1 μg of plasmid and 6 μl of DOTAP in sterile HEPES-buffered saline (150 mM NaCl, 20 mM HEPES) previously incubated at room temperature for 15 min. The plate was incubated for 5 h at 37°C in the presence of 7.5% CO_2. Two hundred microliters of MEM containing 2% FBS was added to each well, and the plate was incubated for 24 to 140 h under the same conditions, before analysis of transient expression by indirect immunofluorescence.

Antibodies. Polyclonal antibodies (PAbs) directed against PV and Mok G were obtained as described elsewhere, by rabbit immunization with purified virus glycoprotein (28). A PAb against EBL1b virus was obtained by mouse immunization with inactivated and purified virus.

Three MAbs directed against PV G were also used. The PVE12 MAb (a gift from M. Lafon) recognizes site II of native G (20). The D1 MAb (immunoglobulin G1 [IgG1] isotype), produced in our laboratory, recognized site III of native but not sodium dodecyl sulfate (SDS)-treated G. The 6A1 MAb (a gift from F. Lafay) recognizes SDS-denatured G protein and more precisely two peptides located downstream from site III, near the COOH-terminal part of the G ectodomain (aa 342 to 433 and 397 to 450) (18).

Immunofluorescence microscopy. Transient expression of G antigens in transfected cells was assessed with and without permeabilization (30-min incubation with 80% acetone on ice followed by air drying). Transfected cells were incubated for 1 h at 37°C with PAb or MAb. They were washed with phosphate-buffered saline (PBS) and incubated for 1 h at 37°C with goat anti-rabbit or anti-mouse fluorescein isothiocyanate-conjugated secondary antibody (Nordic Immunology Laboratories, Tilburg, The Netherlands). Cells were washed, mounted in glycerol, and examined with a Leica inverted fluorescence microscope.

Injection of plasmids into mice. For immunological studies, BALB/c mice were anesthetized with pentobarbital (30 mg/kg of body weight), and 20 μg of plasmid (diluted in PBS) was injected into each anterior tibialis muscle. This was more effective than injection via the quadriceps route (personal observation). Blood was collected for antibody assay of serum on various days by retro-orbital puncture.

IL-2 release assay. Spleens were removed from naive or plasmid-injected BALB/c mice. Splenocytes (1-ml aliquots containing 6×10^6 cells) were stimulated with 0.5 μg of lyssavirus antigen or 5 μg of ConA (Miles) in 24-well plates (Nuclon-Delta; Nunc, Roskilde, Denmark) and cultured as described elsewhere (17, 30) in RPMI 1640 medium (Gibco) containing 10% FBS, 1 mM sodium pyruvate, 1 mM nonessential amino acids, 5×10^{-5} M 2-mercaptoethanol, and 10 mM HEPES buffer (Flow Laboratories). Cells were incubated for 24 h at 37°C in a humidified atmosphere containing 7% CO_2. Under these conditions, the cells producing IL-2 are mainly $CD4^+$ cells (17). IL-2 produced in supernatants of splenocyte cultures was titrated by bioassay with CTLL cells as previously described (29). Cell proliferation was determined in triplicate, based on the uptake of [^{3}H]thymidine (New England Nuclear). The IL-2 concentration is given in units per milliliter, with mouse recombinant IL-2 (Genzyme Corporation, Cambridge, Mass.) used as the reference. CTLL cells grew in the presence of mouse IL-2 and anti-IL-4 antibodies, but not in the presence of IL-4 (up to 10 U/ml) and in the absence of IL-2 (17). So, IL-2 was predominantly detected by this technique.

Antibody assays. Total antirabies IgG or IgG1, IgG2a, and IgG3 isotypes were assayed by enzyme-linked immunosorbent assay (ELISA) with microplates coated with IPRV as previously described (33) with rabbit anti-mouse IgG isotype serum as the secondary antibody (Nordic Immunology Laboratories) and a goat anti-rabbit IgG peroxidase conjugate (Nordic Immunology Laboratories) as the tertiary antibody.

Lyssavirus-neutralizing antibodies were titrated by the rapid fluorescent focus inhibition test (RFFIT) (38) with the previously described modifications (33). Infected-cell supernatants (PV, CVS, and EBL2 viruses) and purified viruses

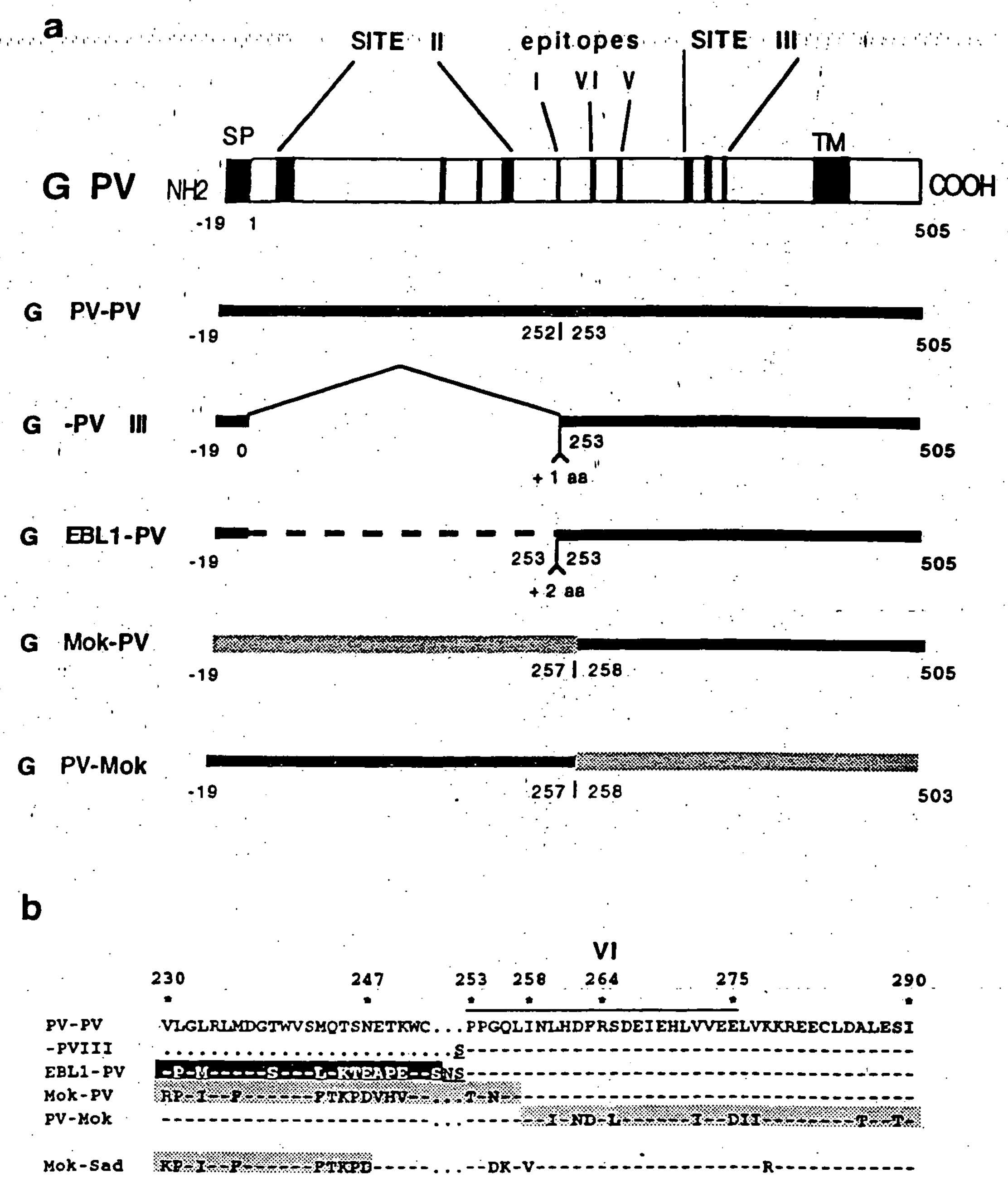

FIG. 1. (a) Schematic representation of homogeneous and chimeric lyssavirus G proteins. The principal antigenic sites and epitopes are shown along the G PV molecule. Numbers give the positions of amino acid residues on the mature protein (signal peptide cleaved). (b) The exact sequences of all proteins including the Mok-Sad construct (26) around the linear region (underlined) carrying epitope VI (aa 264) are shown. Black and gray boxes outline the EBL1 and Mok sequences, respectively. Dashes represent amino acids similar to those in the PV sequence, and dots correspond to gaps.

(Mok and EBL1 viruses) were used. Anti-PV or -CVS antibody titers are expressed in international units per milliliter, with the 2nd International Standard (Statens Seruminstitut, Copenhagen, Denmark) as the reference. For determination of the titers of antibodies against other lyssaviruses, we took the serum dilution causing 50% inhibition of the fluorescent focus rate as having the same VNAb titer as that of the reference assayed against CVS.

Protection test. The protective activity of vaccines and plasmids was determined according to the National Institutes of Health potency test (36). Dilutions of vaccine were injected intraperitoneally (i.p.) into mice on days 0 and 7, whereas plasmids (40 μg) were injected into each anterior tibialis muscle on day 0 only. Mice were then intracerebrally (i.c.) challenged on day 21 with about 30 50% lethal doses (LD_{50}s) of one of the various lyssaviruses (CVS, EBL1b, or EBL2b). The animals were observed for 28 days.

RESULTS

Transient expression of lyssavirus G genes. Plasmids containing homogeneous (pGPV-PV), truncated (pG-PVIII), and chimeric (pGEBL1-PV, pGMok-PV, and pGPV-Mok) lyssavirus G genes were used to transfect Neuro-2a cells. Cell staining by indirect immunofluorescence is shown in Fig. 2 and can be summarized as follows. After transfection with pGPV-PV, antigen was detected with PV PAb (Fig. 2A), PV D1 MAb (Fig. 2B), or PV E12 Mab (not shown), mostly at the cell membrane (similar results were found with nonpermeabilized

cells [data not shown]), and very little antigen was detected with the 6A1 Mab (Fig. 2C). Cells transfected with pG-PVIII were round and completely stained (both cytoplasm and membrane) with PV PAb (Fig. 2D) or 6A1 MAb (Fig. 2F), but not with PV D1 MAb (Fig. 2E). Cells transfected with pGEBL1-PV were stained mostly at the cell membrane with PV PAb (Fig. 2G), PV D1 MAb (Fig. 2H), or EBL1 PAb (Fig. 2I). Cells transfected with pGMok-PV were stained (mainly at the membrane) with PV PAb (Fig. 2J), PV D1 MAb (Fig. 2K), or Mok PAb (Fig. 2L), and very few were stained with the 6A1 MAb (data not shown). Cells transfected with pGPV-Mok were stained with PV PAb (Fig. 2M) or Mok PAb and were round (Fig. 2N). It seems that cell transfection allowed us to distinguish between two types of G antigens: (i) those stained principally at the membrane of cells normal in shape, in particular, with neutralizing MAbs directed to sites II (PV E12) and III (PV D1); (ii) those stained in both the cytoplasm and membrane of round cells, particularly with the MAb (6A1) that recognizes the denatured G molecule.

The kinetics of G protein expression was studied upon transfection of cells with three representative plasmids: pGPV-PV (homogeneous), pGEBL1-PV (chimeric), and pG-PVIII (truncated). pGPV-PV produced G antigens in about 60% of cells when stained with PV PAb, whereas very few cells were stained with the PV 6A1 MAb at any time point (Fig. 3a). About 55% of cells transfected with pGEBL1-PV were stained with PV PAb, and up to 15% were stained with the 6A1 MAb (Fig. 3b), indicating that some G molecules were denatured. Ten to twenty percent of the cells transfected with pG-PVIII were round and stained with PV PAb, whereas 5 to 10% were positive by the 6A1 MAb, indicating that G molecules were denatured (Fig. 3c).

Induction of IL-2-producing cells. The ability of the plasmids pGPV-PV, pG-PVIII, pGEBL1-PV, pGMok-PV, and pGPV-Mok to induce IL-2-producing cells was assayed (Fig. 4). Plasmids with the site III part of PV, whether unfused (pG-PVIII) or fused with any lyssavirus site II part (pGPV-PV, pGEBL1-PV, and pGMok-PV), efficiently induced IL-2-producing cells (240 to 550 mU/ml). This was true even for pG-PVIII, which, however, had only low efficiency for both cell transfection (see above) and antibody induction (see below). For the chimeric plasmids pGEBL1-PV and pGMok-PV, the T-cell response was greater after stimulation with inactivated and purified PV than with the EBL1b and Mok viruses. This was not due to the quality of the purified antigens, because immunization of BALB/c mice with PV, EBL1b or Mok IPRV followed by in vitro stimulation with the same antigen induced similar levels of IL-2 production (PV, 250 mU/ml; EBL1b, 350 mU/ml; and Mok, 400 mU/ml). In contrast, the plasmid pGPV-Mok induced only a weak Th cell response (IL-2 titer, 50 mU/ml), which was similarly produced in vitro after stimulation with either IPRV PV or Mok virus.

Serological assays. The truncated pG-PVIII plasmid did not induce the production of rabies antibodies, when assayed by RFFIT and ELISA. However when IL-2 was injected together with pG-PVIII and then injected alone 7 days later, antibodies were detected on day 21 only by ELISA (data not shown). Thus, the site III part was expressed in vivo and induced a weak production of nonneutralizing antibodies, which was boosted by exogenous IL-2.

In contrast, when the site III part of PV was linked with the homologous site II part, as in pGPV-PV, it displayed strong immunogenicity. A single injection of pGPV-PV plasmid into mice resulted in high levels of VNAbs measured 27 days later against both the homologous PV and CVS viruses and the heterologous EBL2b virus (Fig. 5a). The antibody isotype induced was mainly IgG2a, but a weak IgG1 response was also observed (data not shown). However, the correlation between VNAb titers against PV was stronger with the IgG2a titer (r = 0.974) than with the IgG1 titer (r = 0.71), indicating that VNAbs induced by DNA-based immunization were mainly IgG2a. The VNAb titer against the homologous PV and CVS viruses increased when mice had a booster injection on day 30 and their sera were checked at day 40, but not the VNAb titers against the heterologous EBL2b virus, which remained unchanged (Fig. 5a). Under these conditions, we also demonstrated a relationship between VNAb level induced by pGPV-PV and the protection of mice against an i.c. challenge with CVS: all animals with a VNAb titer (on day 20) above 1.5 IU/ml survived the challenge on day 21 (data not shown). In contrast, no significant amount of VNAb against EBL1b was produced, neither after a single injection nor after a booster injection.

Thus, we investigated the capability of the site III part of PV to carry the heterologous EBL1 site II part, following our previous observation that the chimeric plasmid pGMok-PV induced VNAbs against both PV and Mok viruses (4). A single injection of the chimeric plasmid pGEBL1-PV similarly induced VNAbs against both PV and EBL1b viruses (Fig. 5b). Fourteen days after injection, the titers were 2 IU/ml, and they increased to 17 IU/ml after 80 days. The level of VNAb production against EBL1b was always higher than that against PV, but the difference was not significant. Taken together, these results clearly demonstrate that chimeric G genes encoding the site III part of PV and the site II part of G of other lyssaviruses, such as EBL1b and Mok (4), are very potent inducers of VNAbs against both parental viruses. In contrast, the symmetric pGPV-Mok construct did not induce VNAbs against either the PV or Mok virus (not shown).

Assays of protection against European lyssaviruses. We tested the ability of both the homogeneous pGPV-PV plasmid and the chimeric pGEBL1-PV plasmid to induce protection against an i.c. challenge with viruses representing lyssavirus GTs involved in the transmission of encephalomyelitis in Europe (CVS for GT1, EBL1b for GT5, and EBL2b for GT6). We compared their efficiency with that of a commercially available vaccine (PM strain [GT1]) and a laboratory preparation (PV strain [GT1]) (Fig. 6).

The plasmid backbone (pClneo) did not induce protection against any virus (Fig. 6d, e, and f). In contrast, pGPV-PV protected 70% of BALB/c mice (and 85% of the Swiss mice) against CVS (Fig. 6d), 30% of them against EBL1b (Fig. 6e), and 72% of them against EBL2b (Fig. 6f). Under the same conditions, pGEBL1-PV protected 60% of BALB/c mice against CVS (Fig. 6d), 75% of them against EBL1b (Fig. 6e), and 80% of them against EBL2b (Fig. 6f). Thus, if immunization with any of the two plasmids showed no significant difference in the protection against CVS (GT1) and EBL2b (GT6), the chimeric pGEBL1-PV was far more efficient against EBL1b (GT5) and is clearly the best candidate for protection with DNA-based immunization against the three European lyssavirus genotypes.

Concerning the protection induced by inactivated cell culture vaccines using the PM and PV strains against the same challenges, a human dose of PM vaccine diluted 1/10 protected 80% of mice against CVS (Fig. 6a), 36% of mice against EBL1b (Fig. 6b), and 80% of mice against EBL2b (Fig. 6c). Under the same conditions, 2 μg of IPRV PV protected 100% of mice against both CVS (Fig. 6a) and EBL1b (Fig. 6b) and 85% of mice against EBL2b (Fig. 6c). It seems that for a vaccine dose which protected 80 to 85% of the animals against EBL2b, the PV strain protected 100% of mice and the PM

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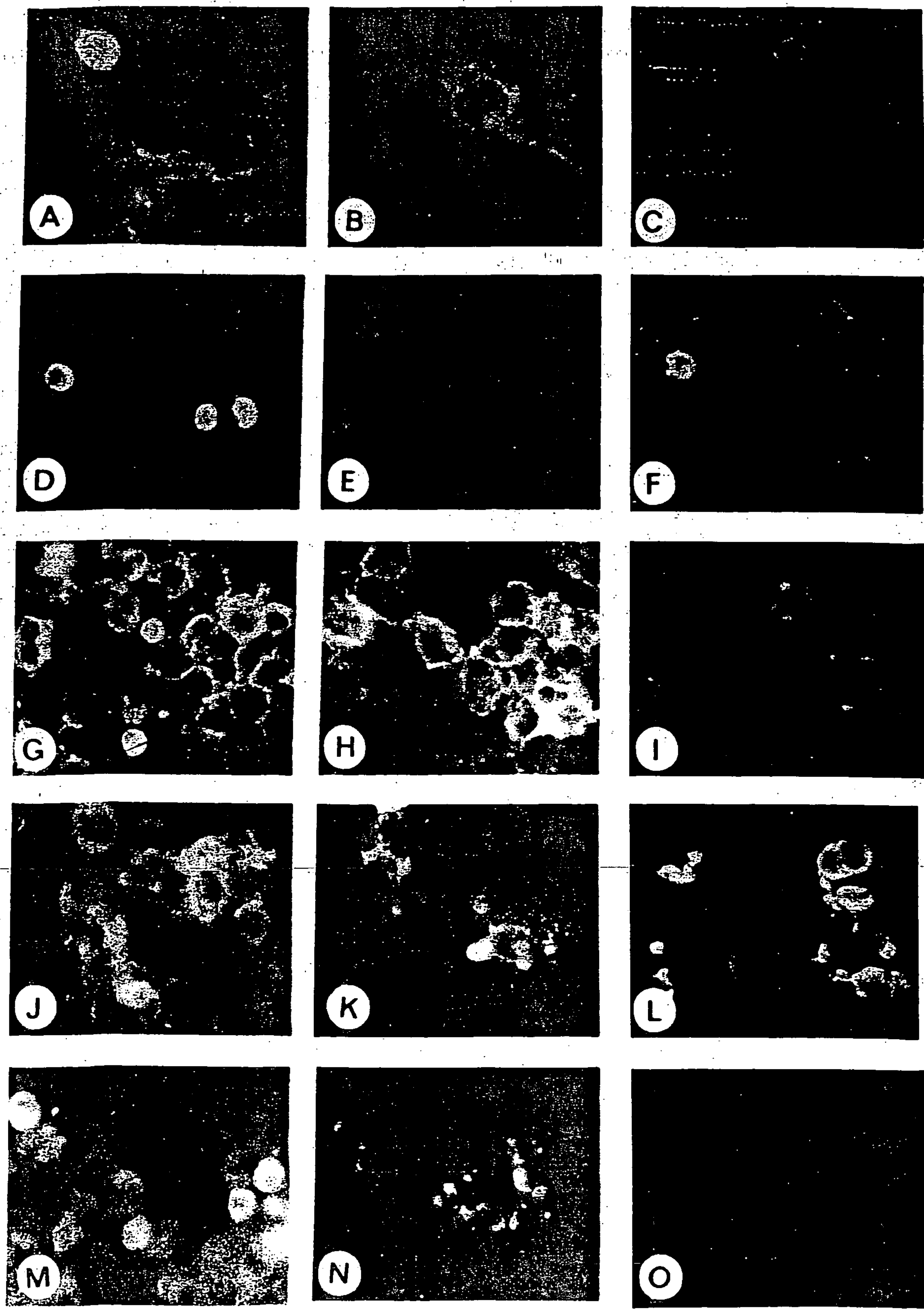

FIG. 2. Indirect immunofluorescence microscopy of lyssavirus G protein production in Neuro-2a cells 48 h after transient transfection with various plasmids: pGPV-PV (A, B, and C), pG-PVIII (D, E, and F), pGEBL1-PV (G, H, and I), pGMok-PV (J, K, and L), and pGPV-Mok (M, N, and O). Forty-eight hours after transfection, cells were permeabilized and stained with antibodies: anti-PV G PAb (A, D, G, J, and M), PV D1 anti-native PV G site III MAb (B, E, H, and K), 6B1 anti-denatured G site III MAb (C and F), anti-EBL-1 G Pab (I), anti-Mok G PAb (L and N), and serum from an unimmunized mouse (O).

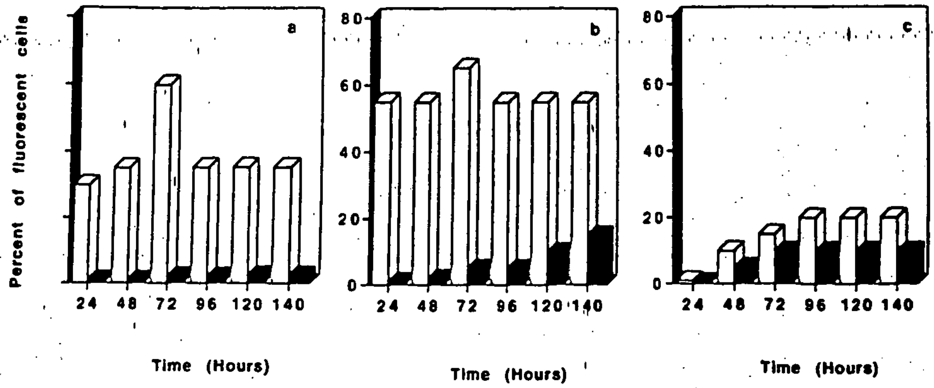

FIG. 3. Kinetic study of antigen synthesis in Neuro-2a cells transiently transfected with pGPV-PV (a), pGEBL1-PV (b), or pG-PVIII (c). Cells were permeabilized at various times and stained with PV PAb (open bars) or the anti-denatured G site III 6B1 MAb (solid bars).

strain protected only 36% of mice against EBL1b. Thus, the PM strain is less effective than the PV strain against EBL1b.

DISCUSSION

The immunogenicity and structure of lyssavirus glycoproteins were assessed by using homogeneous and chimeric plasmids for DNA-based immunization and cell transfection. We investigated the respective functions of G antigenic site II and III parts in the immunological properties of the lyssavirus G proteins and in their protective activity against various genotypes. The only known linear region of the ectodomain, carrying the linear virus-neutralizing epitope VI around aa 264 (10), was used as a flexible hinge to separate the two main antigenic sites: the site II part on the NH_2 side and the site III part on the COOH side. The plasmids tested carried either full-length G genes of a homogeneous (pGPV-PV or pGMok-Mok) or chimeric (pGMok-PV, pGEBL1-PV, or pGPV-Mok) nature or a truncated G gene (pG-PVIII). All of them encoded a signal peptide promoting the protein translocation into the endoplasmic reticulum, as well as the transmembrane and endoplasmic domains for the membrane anchorage.

All plasmids were transiently expressed in transfected Neuro-2a cells and could be classified into two families according to antigen location: (i) those located mainly at the membrane (pGPV-PV, pGMok-Mok, pGEBL1-PV, and pGMok-PV); and (ii) those located inside round cells (pG-PVIII and pGPV-Mok). The G proteins from the first family are normally transported to the membrane and recognized by virus-neutralizing MAbs specific for antigenic sites II and III. This suggests a native folding of these sites as it was evidenced after cell infection with the PV strain (not shown). In contrast, the G proteins from the second family are stained by using PAb or nonneutralizing MAb. For example, the truncated G-PVIII is stained by a MAb recognizing the denatured G protein, but not by a virus-neutralizing MAb directed against site III. This indicates that folding and transport were not correct and possibly induced modifications in cell shape.

It is interesting to consider which of the maturation steps (folding, posttranslational modifications, and oligomerization) are primarily affected in the synthesis of G proteins of the second family. Glycosylation plays a key role in the folding of rabies virus G protein and in its transport from the endoplasmic reticulum to the Golgi apparatus (14, 37). Even though several potential N-linked glycosylation sites exist along the lyssavirus G proteins (12, 39), only Asn^{319} is shared by all of

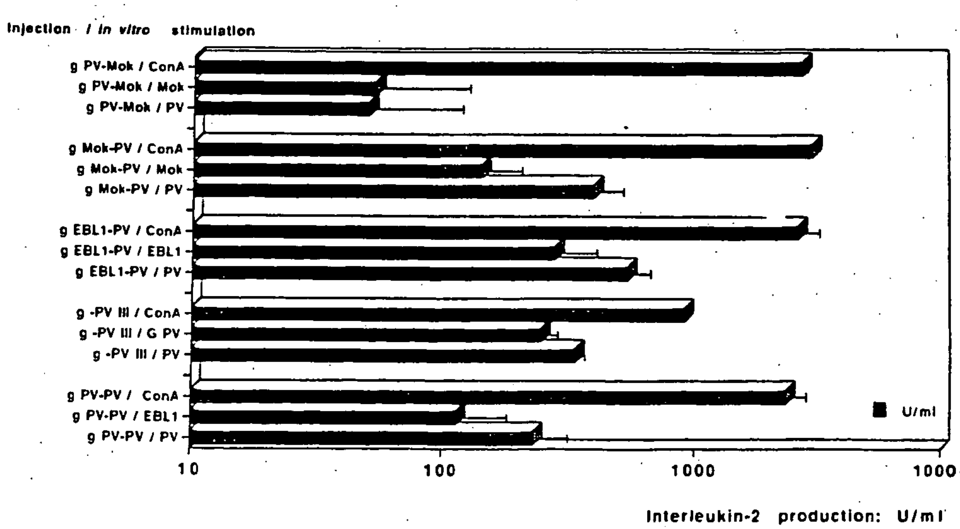

FIG. 4. Induction of IL-2-producing cells by plasmids encoding various lyssavirus G proteins. BALB/c mice (two animals for each plasmid) were injected intramuscularly (50 µl in each anterior tibialis muscle) with 40 µg of plasmid (pGPV-PV, pG-PVIII, pGEBL1-PV, pGMok-PV, and pGPV-Mok). Spleens were removed 21 days later, and splenocytes were specifically stimulated in vitro by inactivated and purified viruses (PV, EBL1, or Mok), G PV, or polyclonally stimulated by ConA. The amount of IL-2 released was then assayed in triplicate by bioassay, and the titers were expressed as units per milliliter.

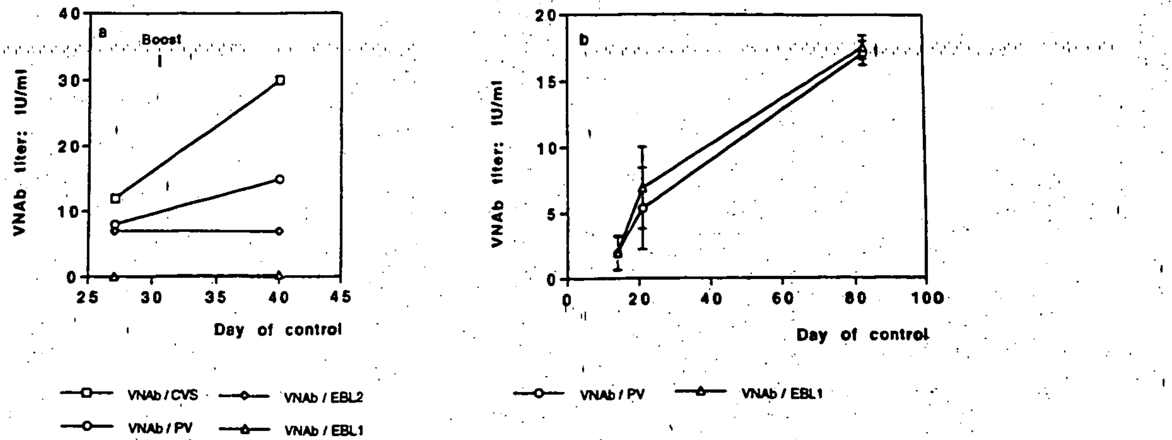

FIG. 5. Induction of VNAb against European lyssavirus GTs by pGPV-PV or pGEBL1-PV plasmid. BALB/c mice were injected with 40 μg of plasmid in the tibialis muscle. (a) Mice received a boost on day 30. Sera (pool of three samples) were assayed on days 27 and 40 for VNAb against viruses of GT1 (CVS and PV), GT4 (EBL1b), and GT5 (EBL2b). (b) Four mice received only one injection of plasmid, and blood samples were collected at various intervals by transorbital puncture. Sera were assayed by RFFIT with PV and EBL1b viruses for VNAb determination.

them, and it is the only N-linked glycosylation site in EBL1 (3), i.e., in the G protein encoded by pGEBL1-PV plasmid. This demonstrates that glycosylation of Asn^{319} is sufficient for correct folding and transport of the G protein (37). Since Asn^{319} is present in pG-PVIII and pGPV-Mok, other factors than glycosylation are probably required for the maturation of these antigens. The cooperative conformation of a lyssavirus site II could be a prerequisite for the correct folding of the PV site III, as demonstrated in both homogeneous (G PV-PV) and chimeric (G Mok-PV and G EBL1-PV) full-length G proteins. However, the symmetrical observation for the Mok site III is not true, since the pGPV-Mok plasmid mainly produced a denaturated chimeric G protein. An alternative hypothesis would be impairment in oligomerization, as supported by the observation that G PV-Mok and G-PVIII induced a low level of antibody or no antibody. Rabies virus G protein oligomerization was shown to be important in both antibody accessibility and immunogenicity (31), and B cells preferentially recognize highly repetitive and organized antigens (2).

Although the antigen produced by the pG-PVIII plasmid does not seem to be correctly folded, it induces IL-2-producing cells in mice at a level similar to that of the plasmids having the site III part of PV fused to any lyssavirus site II (pGPV-PV, pGMok-PV, and pGEBL1-PV). This shows that the Th epitopes are presented and recognized in vivo by Th cells independently of the correct folding of the G protein. IPRV PV was more effective than Mok and EBL1b virus for the stimulation in vitro of splenocytes from mice immunized with the chimeric plasmids pGMok-PV and pGEBL1-PV. In addition, both homogeneous pGMok-Mok (4) and chimeric pGPV-Mok plasmids induced lower levels of Th cells. Taken together, these data suggest that the site III part from the PV strain is more effective for inducing Th cells than the PV strain site II part or the Mok virus site III part.

It is striking that the high levels of IL-2-producing cells induced by immunization with pG-PVIII did not stimulate any antibody production. Only exogenous IL-2 coinjected and boosted 7 days later generated an appreciable antibody level (no VNAb due to the incorrect folding of G-PVIII). This adjuvant effect of IL-2 given as purified protein was previously observed with inactivated rabies antigens according to the same protocol used for IL-2 administration (30), whereas a DNA vector encoding IL-2 was not effective (27). Therefore, exogenous IL-2 appears more effective than endogenous IL-2 from

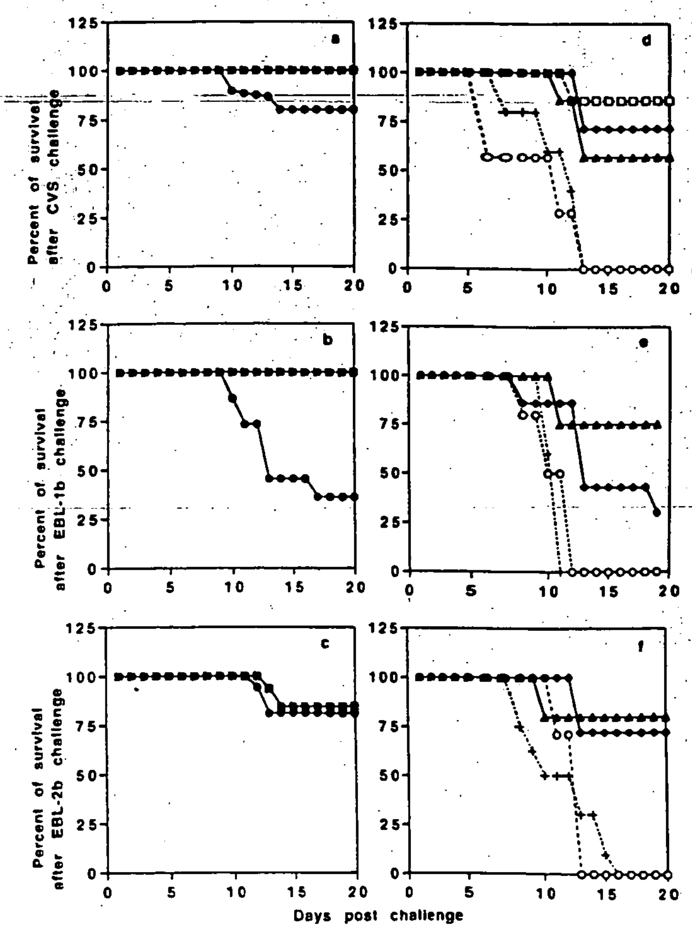

FIG. 6. Comparative protection induced by pGPV-PV and pGEBL1-PV plasmids and rabies virus PM and PV vaccines against CVS, EBL1b, and EBL2b. For immunization with inactivated viruses (a, b, and c), BALB/c mice (nine animals per series) were injected i.p. on days 0 and 7 with 0.5 ml of PM vaccine diluted 1/10 (solid circles) or with 2 μg of IPRV PV (solid squares). For DNA-based immunization (d, e, and f), BALB/c mice (five animals for each plasmid) were injected in the tibialis muscle with PBS (open circles) or with 40 μg of one of the various plasmids pGPV-PV (diamonds), EBL1-PV (solid triangles), and pClneo backbone (crosses). Swiss mice (six animals) were injected with pGPV-PV (open squares). BALB/c and Swiss mice were challenged i.c. on day 21 with about 30 LD_{50}s of CVS (a and d), EBL1b (b and e), and EBL2b (c and f).

any origin, whether produced by Th cells or gene expression from a plasmid.

A key point of this paper is the clear demonstration that lyssavirus glycoprotein molecules can be split into two structurally and immunologically dependent parts: the COOH half carrying site III and anchoring the protein to the membrane and the NH_2 half carrying site II, which stabilizes the conformation of site III. In any combination between site II and site III, the sites are not equivalent. For example, the pGPV-Mok chimera, with an organization similar to that in wild-type G proteins, is expressed in cells, but does not induce VNAb production. In contrast, the site III part of the PV glycoprotein can present various lyssavirus sites II (PV, EBL1, or Mok) with a high level of immunological potency. For example, production of a VNAb, predominantly of the IgG2a subclass, is induced, suggesting that a Th1-like Th cell response is induced by DNA-based immunization (22, 43). A limited flexibility is authorized at the site II-site III junction, at least between aa 257 (pGMok-PV) and aa 247. The latter position marked the junction of a chimeric G gene previously produced by fusion of the site II part of Mok (Eth-16 strain [GT3]) with the site III part of the SAD strain (GT1), homologous to the PV strain (26). Like G PV-PV, G Mok-PV and G EBL1-PV, G Mok-SAD was normally expressed, transported to the cell surface, and immunoprecipitated with MAb recognizing correctly folded antigenic site III (25). However, G Mok-SAD cannot rescue infectious particles, indicating that if the folding of the hybrid was acceptable for immunological properties, it was not sufficient for either oligomerization, attachment to the receptor, or conformational changes necessary to mediate fusion.

We used the lyssavirus chimeric G gene to investigate protection against all lyssavirus GTs circulating in Europe. This concerned not only the rabies viruses (GT1) prevented by classical vaccines, but also the EBLs EBL1 (GT5) and EBL2 (GT6), each of which has been recently subdivided into two very closely related phylogenetic lineages, a and b (1, 6). The protection afforded against EBL1 and EBL2 depends on both vaccine and virus strains. Protection against EBL1a (strain Hamburg) is achieved in mice by the PV strain (21) but not the PM or Flury LEP strain (21, 35). However, the PM strain induces partial protection against EBL2b (a human isolate from Finland) (11). We verified that for doses of PV and PM vaccine which induced similar levels of protection against CVS (80 to 100%) and EBL2b (80 to 85%), the PV vaccine gave greater protection against EBL1b (100%) than did the PM vaccine (36%). This emphasized the advantage of using PV rather than PM as the virus strain for the production of cell culture vaccine. According to protective activity, EBL2b seems to be equidistant from PV and PM, and EBL1b might be closer to PV than to the PM strain. However, according to phylogenetic relationships as well as amino acid conservation in the G ectodomain, strong similarity is observed between PM and PV, while EBL1 and EBL2 strains are equidistant from each of them and from each other (3).

DNA-based immunization with the pGPV-PV plasmid also induced a high VNAb level and gave similar protection (75%) against CVS and EBL2b, confirming an antigenic community between the glycoproteins of GT1 and GT6. However, both the VNAb level and the protection against EBL1b (36%) were weak, contrasting with the 100% protection obtained with PV IPRV. The difference could be due to technical difficulties (injection into the tibialis muscle used for DNA immunization is less reproducible) or to an adjuvant effect of other viral proteins on G immunogenicity for immunization with IPRV.

Because pGPV-PV plasmid did not give satisfactory protection against all European lyssavirus GTs, the chimeric pGEBL1-PV plasmid was constructed to achieve this goal. It induced high levels of VNAb against the two parental genotypes. This indicates that the site III part of PV presents the site II part of rabies-related viruses, and each part maintains its ability to induce specific VNAbs. pGEBL1-PV also induced a high level of protection against EBL2b, similar to that induced by the homogeneous pGPV-PV gene. This indicates that PV site III is mainly involved in protection against EBL2, as previously noted for another chimeric G protein, G Mok-PV (4). The protection induced by pGEBL1-PV mostly correlated with the VNAb production, and the lower level of protection induced against CVS (GT1) could reflect the better immunogenicity of site II (EBL1b sequence [GT5]) compared to site III (PV sequence [GT1]) as suggested by Benmansour et al. (5).

In conclusion, using the capacity of the site III part of PV to correctly present the site II part of Mok (4) or EBL1b virus, we have demonstrated that chimeric G proteins could be used to broaden the range of protection against lyssaviruses. Thus, the simultaneous use of pGMok-PV and pGEBL1-PV chimeric plasmids would be the first experimental vaccine preventing all lyssavirus infection. Work is currently in progress to construct a unique chimeric plasmid offering protection against the six genotypes and to take advantage of the flexibility at the site II-site III junction to carry and potentiate foreign epitopes and promote the lyssavirus G protein as an immunogenic backbone for transdisease vaccines.

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